|CMLS Cellular and Molecular Life Sciences

Review

Antisense RNA gene therapy for studying and modulating biological processes

B. Weiss*, G. Davidkova and L.-W. Zhou

Department of Pharmacology, MCP Hahnemann University, Philadelphia (Pennsylvania 19129, USA),
Fax + 1 215 843 1515, e-mail: weissb@auhs.edu

Abstract. Agents that produce their effects through an antisense mechanism offer the possibility of developing highly specific alternatives to traditional pharmacological antagonists, thereby providing a novel class of therapeutic agents, ones which act at the level of gene expression. Among the antisense compounds, antisense RNA produced intracellularly by an expression vector has been used extensively in the past several years. This review considers the advantages of the antisense RNA approach over the use of antisense oligodeoxynucleotides, the different means by which one may deliver and produce antisense RNA inside cells, and the experimental criteria one should use to ascertain whether the antisense RNA is acting through a true antisense mechanism. Its major emphasis is on exploring the potential therapeutic use of antisense RNA in several areas of medicine. For example, in the field of oncology antisense RNA has been used to inhibit several different target proteins, such as growth factors, growth factor receptors, proteins responsible for the invasive potential of tumor cells and proteins directly involved in cell cycle progression. In particular, a detailed discussion is presented on the possibility of selectively inhibiting the growth of tumor cells by using antisense RNA expression vectors directed to the individual calmodulin transcripts. Detailed consideration is also provided on the development and potential therapeutic applications of antisense RNA vectors targeted to the D2 dopamine receptor subtype. Studies are also summarized in which antisense RNA has been used to develop more effective therapies for infections with certain viruses such as the human immunodeficiency virus and the virus of hepatitis B, and data are reviewed suggesting new approaches to reduce elevated blood pressure using antisense RNA directed to proteins and receptors from the renin-angiotensin system. Finally, we outline some of the problems which the studies so far have yielded and some outstanding questions which remain to be answered in order to develop further antisense RNA vectors as therapeutic agents.

Key words. Antisense RNA; antisense oligodeoxynucleotides; gene therapy; dopamine receptors; calmodulin; oncology; expression vectors; gene expression; dopamine behaviors; naked DNA delivery; antiviral agents; antihypertensives; infectious diseases; antipsychotics.

Introduction

Compounds acting by an antisense mechanism, that is those that interact through Watson-Crick binding with specific sequences on mature messenger RNA (mRNA) or on their precursor mRNAs, include antisense oligodeoxynucleotides [1–6], antisense RNA [2, 7–11] and ribozymes [12–14]. Studies performed over the past several years have shown that an antisense strategy can be used to solve diverse problems in basic biomedical

---

* Corresponding author.

research and have also suggested the possibility that this strategy may be used for developing novel therapeutic agents. Antisense oligodeoxynucleotides have been most extensively studied and have been the subject of several recent reviews and monographs [1–6]. Therefore, they will not be the major object of the present review. Rather, this review will focus on the more recent studies of the properties and applications of antisense RNA, which we believe offer several distinct advantages over the antisense oligodeoxynucleotides. We will summarize the evidence showing how these highly specific alternatives to traditional pharmacological antagonists can be used to block the functions of proteins expressed by mammalian cells in vitro and in vivo and how they ultimately may be developed into clinically useful agents for treating relevant diseases in humans. In particular, we have included studies from our own laboratory wherein we have designed vectors expressing antisense RNAs which may have applications for studying and treating certain neuropsychiatric disorders and particular types of cancer.

Types of antisense approaches

Although the basic principle underlying the development of all antisense compounds is similar, that is complementary pairing between the antisense compound and its specific target, several different types of antisense approaches have been developed depending on the nature of the antisense compound. The different types of antisense compounds may act at various stages of the synthesis of a biologically active target protein, but all are designed to ultimately inhibit the function of the protein. The different types of antisense approaches employed to date are briefly presented below.

One type of antisense approach is the use of antisense deoxyribonucleic acids. Antisense oligodeoxynucleotides are relatively short (usually 15–25 bp), single strands of deoxyribonucleotides, which are designed to be complementary to discrete sites on a specific targeted messenger RNA forming DNA:RNA hybrids. The binding of the antisense agent to the transcript, if effective, inhibits the translation of the encoded protein. Oligodeoxynucleotides have probably been the most extensively studied antisense compounds and have already been used with success in human clinical trials [15, 16]. It should be pointed out that oligodeoxynucleotides have also been used to inhibit gene transcription by other than true 'antisense' mechanisms. These approaches include (i) the 'antigene' approach in which the oligodeoxynucleotides are designed to bind to DNA directly and, by forming a triple-stranded helix, to inhibit transcription [7]; and (ii) transcription factor decoys, which are oligodeoxynucleotide duplexes designed

to bind to transcription factors and, by sequestering them, inhibit their function [17].

Another type of antisense approach utilizes ribonucleic acids which, like antisense oligodeoxynucleotides, bind by complementary interactions with a specific target mRNA (in this case forming RNA:RNA hybrids) and subsequently block one of the several steps involved in the translation of a targeted protein. This approach, which will be the major subject of this review, can use naked DNA-containing genes that express antisense RNA, or can use expression vectors that continuously synthesize antisense RNA inside the cell (this approach will be referred to as antisense RNA gene therapy). The basic mechanism involved is similar to that used in gene therapy except that an antisense RNA rather than a sense RNA is produced by the inserted transgene. An advantage of antisense RNA gene therapy over the more widely employed sense gene therapy is that the antisense RNA approach requires a knowledge and synthesis of only a small portion of the total gene sequence. Moreover, the promoter and other regulatory sequences on the gene need not be known. An interesting related approach is the use of transgenic mice expressing antisense RNA [14, 18]. Other types of antisense RNA strategies include the use of ribonucleic acids with catalytic activity, termed ribozymes, which cleave messenger RNA [12, 19], and peptide nucleic acids, which have been shown to hybridize in a sequence-specific fashion to single-stranded RNA or DNA [20].

Advantages of antisense approaches over conventional pharmacotherapy

As reviewed elsewhere [21], several characteristics make antisense compounds more attractive than conventional pharmacological antagonists for inhibiting the function of specific proteins. Antisense DNA and RNA have extremely high specificity for their target, a degree of specificity which cannot ordinarily be achieved using conventional pharmacological antagonists. Since the antisense compounds inhibit the function of a given protein at the nucleic acid level, they can be used to uncover the role of different transcripts encoding the same protein or the role of closely related subtypes of proteins such as neuroreceptors. In addition, antisense compounds can be easily designed and only require information on the nucleic acid sequence encoding a given protein without prior knowledge of the function of that protein. This may be especially useful for uncovering the function of newly discovered genes. Finally, there is evidence suggesting that antisense compounds (oligodeoxynucleotides and antisense RNA) may inhibit the synthesis of a small pool of newly formed receptors,

which may constitute the pool of functional receptors responsible for mediating their biological effects [22, 23]. By inhibiting the synthesis of receptor proteins rather than blocking the interaction between the receptors and their neurotransmitters, as conventional pharmacological antagonists do, the antisense compounds may not induce compensatory elevations in the levels or the biological functions of the receptors, a problem which arises from the long-term application of certain traditional pharmacological receptor antagonists (see below).

Antisense compounds, however, unlike conventional pharmacological antagonists, have been used in vivo only recently, and there is relatively little information about the possible long-term consequences from their repeated use. Although antisense compounds have yielded promising results in initial trials in animals and humans, there is little knowledge about their possible toxicities. Further, since antisense agents act by inhibiting protein synthesis and, therefore, have an effect only when the proteins are turning over, they may have a longer onset of action than the pharmacological antagonists. This is particularly true if the target protein has a long half-life. Most receptor proteins, however, have a relatively short half-life.

Within the past few years it has been found that several mammalian genes have naturally occurring antisense RNA species, and it has been proposed that these antisense RNAs are mediators of gene expression [24]. This evidence suggests that antisense compounds may be a safe approach which can be used to develop therapeutic agents. The antisense compounds, however, especially vector-generated antisense RNA, may be more difficult to standardize than the traditional pharmacological drugs. Since they would be produced inside the cell by an expression vector, the level of synthesized RNA would be dependent upon many factors which are difficult to control, such as the strength of the promoter in a particular cell type.

Comparison between the properties of antisense oligodeoxynucleotides and antisense RNA

There are several differences in the properties and application of antisense oligodeoxynucleotides and antisense RNA expression vectors. One advantage of antisense oligodeoxynucleotides is that they can be readily synthesized and therefore are readily available from commercial sources. By contrast, constructing recombinant vectors expressing antisense RNA requires more specialized biotechnology and can sometimes be time-consuming. In addition, since antisense oligodeoxynucleotides are manufactured using DNA-synthesizing equipment, these compounds may be more easily stan-

dardized than antisense RNA, which is transcribed intracellularly from a vector, an issue which might be of importance if these reagents are developed as therapeutic drugs. Another important advantage antisense oligodeoxynucleotides have over antisense RNA is that the oligodeoxynucleotides can be chemically modified to increase their solubility, specificity, stability, potency and penetration into cells. For example, phosphorothioate oligodeoxynucleotides, which are currently the most widely used oligonucleotide derivative, are relatively resistant to nucleases and, therefore, are more stable in biological media, although some of these oligonucleotides have been shown to have significant toxicity [2]. Of the many other chemically modified oligonucleotides that have been studied, peptide nucleic acid analogs are particularly interesting, as these agents have been shown to bind to both single-stranded and double-stranded nucleic acid moieties with high affinity and sequence specificity. They are also quite stable to nucleases and peptidases [20].

Expression vectors producing antisense RNA have major advantages over the oligodeoxynucleotides in that, since the vector can synthesize the antisense RNA continuously inside the cell after a single administration, it would have a longer duration of action. In addition, the antisense RNA-generating vectors offer the possibility of incorporating cell-specific promoter elements, which will allow the expression of the antisense RNA only in certain target cells. These issues are especially important in the development of therapeutic agents (see below for a model by which one may selectively target individual cell types).

Other comparisons of the characteristics of antisense oligodeoxynucleotides and antisense RNA have been discussed in detail in several earlier reviews [2, 11, 21]. Both types of antisense compounds may be suitable for studying the effects of inhibiting the synthesis of target proteins; the choice of compound depends on the aim of the study and the technical expertise of the laboratory. Often it is appropriate to perform the initial studies with antisense oligodeoxynucleotides because they can be readily synthesized. In addition, using oligodeoxynucleotides it is more feasible to examine simultaneously several oligodeoxynucleotides complementary to different sites on the target mRNA in order to determine which region might be more susceptible to antisense inhibition. If these experiments yield successful results, they might serve as the basis for the development of antisense RNA directed to the region of the targeted mRNA which has been found to be susceptible to antisense inhibition. Experience from our laboratory with antisense oligodeoxynucleotides targeted to the transcripts encoding the dopamine receptor subtypes [23, 25, 26] and the individual calmodulin transcripts [27, 28] suggested that the main disadvantage of the use

of antisense oligodeoxynucleotides is the need for multiple administration. This was the reason to employ expression vectors synthesizing antisense RNA to the transcripts encoding the D2 dopamine receptor [2, 29] and calmodulin [29, 30] (see below for more details of these studies).

A major advantage of the use of antisense RNA is the long-term expression of the antisense compound inside the cell after a single administration. As mentioned earlier, since the antisense RNA is produced by an expression vector, it is possible to target the expression vector only to certain cell types and to incorporate a promoter which allows inducible expression of the antisense RNA. These properties would be especially useful when developing therapeutic agents to block the expression of abundantly expressed targets, such as the dopamine receptors or calmodulin, which are expressed in different regions where they mediate distinct functions. A difficult problem associated with the use of antisense RNA expression vectors, however, is the poor penetration and uptake of the expression vectors into the tissues and cells in comparison with the antisense oligodeoxynucleotides. For example, using a fluorescently labeled antisense oligodeoxynucleotide to the D2 dopamine receptor, we found that after a single injection into the lateral ventricle, the oligodeoxynucleotide penetrated rapidly into many brain regions, including the corpus striatum, septum, hippocampus, hypothalamus, cerebral cortex, nucleus accumbens, substantia nigra and cerebellum [31]. However, studies of the distribution of antisense RNA vectors in brain showed a far slower and less invasive spread from the injection site [32]. Although in these studies the antisense RNA expression vector was administered intrastriatally and

not intracerebroventricularly, the distribution of the vector found by in situ polymerase chain reaction (PCR) was less than expected considering the injection volume (5 µl into one striatum) and the amount of plasmid vector DNA (25 µg).

Delivery, expression and targeting antisense RNA expression vectors

A large body of information concerning the properties of vectors suitable for the delivery and expression of nucleic acids has been derived from studies in which the aim was to develop gene therapy for treating human disorders. Much of this information can be adapted for antisense RNA gene therapy. Several approaches may be utilized in order to effectively deliver and express the antisense RNA inside the cell. These plans of attack, which have been the subject of several recent reviews [33–36], may be generally divided into the use of naked DNA that expresses an antisense RNA or the use of RNA-generating vectors, the latter using a viral, nonviral or tailored approach to gain access to cells. Naked DNA, while relatively simple to design and administer, is likely to have a short duration of action and, unlike vectors generating antisense RNA, cannot readily be targeted to individual cell types. The main characteristics of the different vectors that may be suitable to express sense or antisense RNA are summarized in table 1.

Viral vectors are defective viruses that retain the inherent tropism of the viruses for certain cell types and utilize strong promoters for expression of the recombinant sequence. However, in many cases they retain characteristics of the wild-type viruses, which may cause

Table 1. Characteristics of vectors suitable for the expression of antisense RNA.

Vector Type	Advantages	Disadvantages
Retrovirus	accommodates large foreign sequences; transduces many replicating cells	difficult production of high titer virus preparations; random integration into the host genome
Lentivirus	transduces nondividing cells; lack of expression of immunogenic viral proteins	inherent pathogenicity of wild-type HIV virus
Adenovirus	accommodates large foreign sequences; transduces many replicating and nonreplicating cells; easy purification of large-titer virus preparations; episomal	induction of strong immune response in the host
Adeno-associated virus	transduces replicating and nonreplicating cells; relatively non-toxic; small genome	accommodates small foreign sequences; requires helper virus
Herpes simplex virus	accommodates large foreign sequences; transduces nerve cells	marked neurotoxicity
Nonviral plasmid DNA	ease of production; transfects dividing and nondividing cells; does not integrate into the host genome	low transfection efficiency; unstable transgene expression
Cationic lipids	readily available; large variety suitable for different cell types; support high efficiency of transfection	toxicity at high doses
Molecular conjugates	selective targeting to certain cells or subcellular structures	large size; nonspecific binding; difficult synthesis

undesired effects in their host. Retroviral vectors have been one of the key vector types for gene therapy because of their wide host range. However, their use has been limited by the difficulties in purifying high-titer recombinant viruses. An additional disadvantage has been their ability to integrate into the host genome, thus creating the risk of insertional mutagenesis. Until recently the use of retroviral vectors was limited only to dividing cells, but a new human immunodeficiency virus-based vector has been developed which has the ability to target terminally differentiated neurons of the central nervous system [37]. Major advantages of the adenovirus-based vectors have been their ability to accommodate long genes, to transduce many dividing and nondividing cell types, to persist in an episomal state in the host and to be purified to large titer preparations rather easily. These vectors, however, have been reported to induce a strong immune responses in the host [38, 39]. Although steps have been taken to obtain vectors deleted of the genes that induce an immune response, this problem is still not entirely solved [40, 41]. A promising vector system for the delivery of genes into both dividing and nondividing cells is based on the defective adeno-associated viruses [42, 43]. At present the use of this system is restricted by the relatively short recombinant sequence which can be inserted and the technical problems in producing large quantities of viral vectors [44]. Herpes simplex virus type 1-based vectors are particularly suitable for delivering foreign genes into neurons because the herpes simplex virus can establish long-term latency in neurons, but these types of vectors have been reported to be cytotoxic [45, 46].

Nonviral vectors contain mammalian expression plasmids and do not penetrate into cells as efficiently as the viral vectors do, but do not have as many side effects associated with their use. For example, they lack the inherent pathogenicity of viral vectors. Their production is relatively easy as compared to the production of high-titer viral vectors. The nonviral vectors have been shown to penetrate into both dividing and nondividing cells. Moreover, they have not been shown to integrate into the genome in vivo, and therefore are not likely to create a risk of insertional mutagenesis. A great number of cationic lipids and polymers have been complexed with the plasmid DNA in order to protect it from the action of nucleases and to enhance the entry of plasmid DNA into the cells in vitro and in vivo [47]. Although the cationic lipids are relatively safe, at high doses they may elicit inflammatory or toxic reactions [48]. The tailored approaches include vectors that have been designed so that the vector is targeted only to specific cell types, which increases their uptake into these cells [49]. These approaches involve the construction of molecular conjugates between nonviral or viral vectors and low molecular weight effector molecules which usually target the entire conjugate to a cell surface receptor. For example, antibodies can be used as cell-targeting ligands. The molecular conjugates have also been constructed to contain effectors, such as adenovirus, which facilitate the escape of the vector DNA from the endosome [50], or molecules that enhance the entry of the DNA into the cell nucleus [51]. Some difficulties associated with this type of approach are the large size of the molecular conjugates and the immunogenicity of the targeting ligands.

A problem with all vectors, more evident with the nonviral vectors, is that the expression of the antisense RNA disappears with time. This, however, is a property of traditional pharmacological agents as well, almost all of which have a limited duration of action. Indeed, nonviral vectors may have a duration of action that is far longer than most conventional drugs (see below). Of course, in some cases a short duration of action is a desirable feature. Ideally, though, one would have the ability to determine and alter the duration of action of the agent to suit the particular therapeutic regimen. Such an option may be achievable in an antisense RNA delivery system if an inducible promoter is incorporated into its sequence and if this promoter is activated by an exogenously administered transcription activator that itself has a short duration of action (see the last section in this review on outstanding problems and questions related to the development of antisense RNA expression vectors).

The main characteristics of the different vectors presented above have determined the choice of vectors in the antisense RNA experiments performed to date. For example, from table 2 on the in vivo application of antisense RNA in oncology (see below), it can be seen that nonviral expression plasmids have been frequently used because they are easier to use than the viral vectors and do not have as many toxic effects. However, for certain cell types that proliferate very rapidly, such as leukemic cells, retroviral vectors are the most appropriate choice. In contrast, these retroviral vectors would not be suitable to deliver antisense RNA into nondividing normal brain tissue.

Potential therapeutic applications of antisense RNA expression vectors

Although there was originally skepticism toward the possibility of inhibiting gene expression using antisense RNA, in the past several years numerous reports have demonstrated the application of antisense RNAs to study biological processes, and there are even suggestions of their potential utility as therapeutic agents in neurology, psychiatry, cardiology, infectious diseases and oncology. Most of the studies on the use of anti-

Table 2. Potential therapeutic application of vectors expressing antisense RNA in oncology.

Target protein	Antisense RNA vector type	Experimental model*	Antisense effects	Reference
Human papilloma virus 16 (HPV16)	adenoviral, Ad5CMV	human cervical cancer cells harboring HPV16, SiHa	suppression of cell growth in vitro; inhibition of tumorigenicity in mice	53
Insulin-like growth factor receptor type I (IGF-IR)	plasmid with Zn-inducible, metallothionein-1 promoter	rat glioma cells, C6	inhibition of cell growth in rats	54
Insulin-like growth factor receptor type I (IGF-IR)	plasmid with Zn-inducible, metallothionein-1 promoter	rats injected subcutaneously and intracranially with nontransfected C6 glioma cells	subcutaneous injection of IGF-I antisense RNA-transfected C6 glioma cells into rats prevents the formation of subcutaneous or brain tumors induced by nontransfected C6 cells	55
Insulin-like growth factor receptor type I (IGF-IR)	episomal plasmid	hepatocarcinoma cells, LFCI2 A that produce tumors when injected subcutaneously into syngeneic Commentry rats	IGF-I antisense RNA-transfected LFCI2 A cells are poorly tumorigenic and induce reduction in the size of already established tumors	60
Insulin-like growth factor receptor type I (IGF-IR)	plasmid with inducible *Drosophila* heat shock promoter	rat glioma cells, C6	inhibition of IGF-1-mediated growth in monolayers and clonogenicity in soft agar; subcutaneous injection of IGF-I antisense RNA-transfected C6 glioma cells into rats causes complete regression of previously established wild-type C6 tumors	56
Insulin-like growth factor receptor type I (IGF-IR)	plasmid	murine metastatic carcinoma cells, H-59	reduced expression of IGF-IR and inhibited responsiveness to IGF-IR in vitro; suppression of the metastatic potential of the cells in vivo	57
Insulin-like growth factor receptor type I (IGF-IR)	plasmid with Zn-inducible, metallothionein-1 promoter	rat prostate adenocarcinoma cells, PA-III	inhibition of the expression of IGF-IR, tissue-type and urokinase-type plasminogen activators in PA-III cells; suppression of tumor growth in mice	59
Ets-family transcription factor E1AF	plasmid	human oral squamous cell carcinoma cells, HSC3	suppression of the invasive potential in three-dimensional raft cultures; suppression of the invasive potential into nude mice	68
Urokinase plasminogen activator receptor, uPAR	plasmid	human epidermoid carcinoma cells, HEp3	receptor-deficient cells enter a state of survival without signs of progressive growth	69
Secreted protein acidic and rich in cysteine, SPARC	plasmid	human melanoma cells	decrease in the in vitro adhesive and invasive capacities of tumor cells; complete abolition of in vivo tumorigenicity	70
Calmodulin	plasmid	rat pheochromocytoma, PC12 cells	decrease in the rates of cell proliferation; appearance of neurite outgrowth	29
Gastrin	retroviral	human colon cancer cells	suppression of the proliferative and tumorigenic potential of tumor cells	162
Vascular endothelial growth factor, VEGF	plasmid	nude mice implanted subcutaneously with antisense-VEGF-containing rat C6 glioma cells	suppression of the proliferative potential and reduced formation of vessels in vivo	63
BCR-ABL gene product of the Philadelphia chromosome, P210	retroviral	murine B10 cells; human erythroleukemia cells, K562	inhibition of the proliferation of B10 and K562 cells in vitro	73

Table 2. Continued.

Target protein	Antisense RNA vector type	Experimental model*	Antisense effects	Reference
Phosphoprotein p18	retroviral	human erythroleukemia cells, K562	growth arrest at lower saturation density, loss of serum independence, loss of anchorage-independent growth in vivo; inhibition of tumorigenicity of leukemic cells in vivo in the SCID mouse	74
O^6-Methylguanine-deoxyribonucleic acid methyltransferase, MGMR	plasmid with Zn-inducible metallothionein-1 promoter	rat glioma cells, C6	repression of the resistance to cytocidal effect of nitrosoureas	79
MDR1 specific P-glycoprotein	retroviral	vincristine- and adriamycin-resistant KBV200 cells	cells transfected with antisense RNA to the product of the MDR1 gene have an increased sensitivity to adriamycin and vincristine	163
Cyclin G1, CYCG1	retroviral	human osteosarcoma cells, MG-63	inhibition of cell growth in culture and induction of apoptosis	75
Cyclin G1, CYCG1	retroviral	human osteosarcoma cells, MNNG/HOS implanted subcutaneously into nude mice	inhibition of cell growth in nude mice	76
Protein kinase Cα	plasmid	human glioblastoma cells, U-87	inhibition of cell growth in vitro and abolition of in vivo tumorigenicity	164

CMV, human cytomegalovirus; SCID, severe combined immune deficiency.
*Only the major experimental model is indicated in the table because most of the studies include both in vitro cultured cancer cells and animal models. Studies in which the major experimental model is an in vitro cultured cell line should involve confirmation of the effects of the antisense RNA transfected cells in vivo in an appropriate animal model, since the ultimate goal is the development of therapeutically useful antisense RNA agents.

sense RNA expression vectors have been performed in in vitro preparations [2]. Indeed, there have been hundreds of reports in the scientific literature of the more general use of antisense RNA vectors as tools to investigate the functions of different proteins in vitro, and several reviews have been already published on this subject [2, 7–10]. Therefore, we will concentrate our attention on the effects of antisense RNA expression vectors in vivo, paying particular attention to their potential clinical use.

Oncology

The potential utility of antisense RNA expression vectors for gene therapy of oncologic diseases has been the subject of numerous investigations (table 2). Some reasons for this intense interest in the use of antisense RNA in the area of oncology are that oncologic conditions are difficult to treat, that there are serious side effects associated with the use of conventional therapeutic approaches based on radiation and chemotherapeutic drugs, that these treatment modalities are toxic to normal as well as to tumor cells and that the tumor cells often develop resistance to the therapy. The antisense RNA approach allows the investigators not only to study the function of newly discovered tumor proteins but also to explore the suitability of antisense RNAs as potential therapeutic agents which target these tumor-inducing proteins. In addition, in untreatable oncologic conditions the experimental use of antisense RNA-based therapeutic approaches may be more easily justified. The general paradigm that has been undertaken in performing these studies has been a combined in vitro/in vivo approach. The initial studies have usually been carried out in suitable cell lines expressing the targeted tumor antigen and subsequently extended in vivo in order to evaluate the efficacy in appropriate animal models. From table 2 it can be seen that a number of different types of proteins associated with the malignant phenotype of cancer cells have been targeted with antisense RNA, such as receptors for growth factors (e.g. the receptor for insulin-like growth factor), growth factors themselves (e.g. vascular endothelial growth factor), viruses (e.g. human papilloma virus), transcription factors (e.g. ets-family transcription factors), products of abnormal gene rearrangement (e.g. the BCR-ABL product of the Philadelphia chromosome), enzymes (e.g. enzymes conferring resistance to antitumor drugs and enzymes involved in the proliferative potential of tumor cells) and proteins directly involved in cell cycle progression (e.g. cyclins and phosphoprotein p18). In addition to these targets, many other proteins expressed by tumor cell lines have been targeted using antisense RNA in in vitro studies of the biology of tumor cell growth [10, 11, 52], but these studies are beyond the scope of the present review. Some examples of the potential therapeutic use of plasmid designed to express antisense RNA for the

purpose of inhibiting tumor cell growth are discussed below. To explore the potential of antisense RNA in the treatment of cervical cancer, an adenoviral vector was used to transcribe RNA antisense to the transcripts of the E6 and E7 genes of human papilloma virus (HPV) 16 [53]. These viral gene products were chosen as targets because of the well-established role of HPV in the development of cervical cancer. A recombinant adenoviral cassette, containing a human cytomegalovirus (CMV) promoter and the entire E6/E7 region of HPV inserted in the antisense orientation, was transduced into SiHa human cervical cancer cells. The antisense vector-infected SiHa cells evidenced the presence of HPV 16 E6/E7 antisense RNA and had markedly decreased growth rates. Subsequently, the HPV 16 E6/E7 antisense RNA-infected cells were injected into nude mice. These cells demonstrated loss of tumorigenicity, suggesting that this might be a suitable approach to the therapy of HPV 16-positive cervical cancer.

Encouraging results were obtained in a series of studies in which the insulin-like growth factor receptor type 1 in tumors was targeted using antisense RNA (IGF-IR) [54–60]. Insulin-like growth factors are critical regulators of cell growth, an action that is mediated by their binding to the IGF-IR. The IGF-IR receptor is overexpressed by glioblastomas, which are tumors with a highly malignant course and are very difficult to eradicate. In most of the aforementioned studies [54–56, 58, 60], different types of plasmid vectors expressing IGF-IR antisense RNA were initially transfected into rat C6 glioma cells. This treatment caused the inhibition of glioma cell proliferation in vitro and subsequently in vivo after injection of the antisense-transfected glioma cells into rats. More important, one of these studies demonstrated that the IGF-IR antisense-transfected C6 glioma cells, upon injection into rats, prevented the formation of tumors induced by the injection of non-transfected C6 glioma cells into the same animal. The antisense-transfected glioma cells also caused regression of already established brain glioblastomas when injected at a point distal to the tumor, effects which were the result of a glioma-specific immune response [55]. The induction of regression of already established C6 glioma tumors in rats after injection of anti-IGF-IR antisense RNA-transfected C6 glioma cells was also documented in a later study [56], thus providing substantial support for the possible role of IGF-IR antisense RNA in the therapy of glioblastomas. In another study, it was shown that IGF-IR antisense RNA not only prevented the growth of primary tumors but also prevented the formation of metastasis [57]. The highly metastatic murine carcinoma H-59 cells were transfected with a plasmid vector expressing IGF-IR antisense RNA. When injected in vivo, these cells did not give rise to metastases under conditions which al-

lowed wild-type or control transfectants to form multiple hepatic and pulmonary metastases. Since IGF-IR is highly expressed by malignant gliomas, which are usually very difficult to manage, the use of antisense RNA may be a suitable adjunct to the existing therapeutic approaches.

Type I insulin-like growth factor receptors are also overexpressed by prostate cancer cells. These cells selectively metastasize to bone, an environment rich in insulin-like growth factors which provides support for further prostate cancer cell growth. In an attempt to limit the growth of prostate cancer, rat prostate adenocarcinoma cells (PA-III) were stably transfected with a plasmid vector producing IGF-IR antisense RNA under the control of a zinc-inducible metallothionein promoter [59]. Treatment with zinc resulted in a reduction of the expression of both tissue-type plasminogen activator and of urokinase-type plasminogen activator by PA-III cells. These effects were also accompanied by a dramatic reduction in the levels of the endogenous IGF-IR mRNA, thus suggesting that the reduction in the levels of these activators was a consequence of the induction of IGF-IR antisense RNA in the transfected cells. Furthermore, mice injected with the IGF-IR antisense-transfected PA-III cells either developed tumors 90% smaller than those of the controls or remained tumor-free for a relatively long period of time. These results suggested that IGF-IR might be a suitable target for antisense RNA treatment of prostate cancer, which has been ranked as one of the leading causes of death from malignancy in men in the United States.

Another means by which one may develop an effective therapy for cancer involving solid tumors is by inhibiting tumor angiogenesis, as vascularization is critical for tumor growth and progression [61]. For example, one event that accompanies the progression of gliomas is an increase in angiogenesis. Low-grade gliomas are moderately vascularized tumors, whereas high-grade gliomas show prominent microvascular proliferations and areas of high vascular density [62]. Vascular endothelial growth factor (VEGF), a powerful mitogen for vascular endothelial cells that promotes angiogenesis in solid tumors, was chosen as the target to inhibit using antisense RNA [63]. Rat C6 glioma cells were transfected with a plasmid vector expressing antisense RNA to VEGF and were subsequently observed to express reduced levels of VEGF in culture under hypoxic conditions (hypoxia rapidly induces VEGF in tumor cells). When implanted subcutaneously into nude mice, the growth of the VEGF-antisense RNA-transfected cells was greatly inhibited compared with the control cells, despite the fact that they showed a faster division time in vitro. Analysis of the tumors resulting from the antisense RNA-transfected cells revealed that, in addition to having a relatively small overall size, they had

fewer blood vessels and a higher degree of necrosis [63]. This study suggested that VEGE might be suitable target for antisense RNA inhibition of tumor angiogenesis. In addition, this approach might be a suitable alternative to the currently developed antiangiogenic drugs. For example, two new antiangiogenic drugs that have been widely publicized because they have shown encouraging results in experimental animals are endostatin and angiostatin [64–67]. Studies in mice have shown that angiostatin inhibits the development of blood vessels in already established tumors, whereas endostatin inhibits the formation of new tumors. Although these drugs may be promising, their potential toxic effects in humans are not yet known. In addition, concern has been raised whether drugs that inhibit the formation of new tumor blood vessels in tumors might impair the ability of the body to heal wounds. In this respect, an antisense RNA agent would have an advantage if it could be selectively delivered and expressed only in the tumor cells. There is also the possibility of employing an antisense RNA agent as an adjunct to other antiangiogenic therapies for more effective eradication of the tumor cells.

Proteins linked to the invasive potential of tumor cells are also suitable targets for antisense RNA inhibition, as acquisition of an invasive/metastatic potential is a key event in tumor progression. In one study, antisense RNA was used to inhibit the function of an ets-family transcription factor, E1AF [68]. This target was chosen because it had been reported that E1AF can upregulate the transcription of matrix metalloproteinase genes and can confer an invasive phenotype on human cancer cells. An E1AF antisense RNA expression plasmid vector was transfected into a human oral squamous cell carcinoma-derived cell line (HSC3), which manifests high levels of both E1AF and the matrix metalloproteinase genes MMP-1 and MMP-9 [68]. The antisense-transfected cells showed a lower invasive potential in three-dimensional raft cultures and in nude mice. This was likely due to the effects of the antisense RNA, as it was accompanied by decreased mRNA and decreased levels of the MMP-1 and MMP-9 proteins in the squamous cell carcinoma HSC3 cells. It should be emphasized, however, that when targeting transcription factors that are not tumor cell-specific, it would be useful to selectively inhibit the function of the transcription factor in the tumor cells, while sparing the normal cells.

Another protein linked to the invasive potential of tumor cells which has been targeted using antisense RNA is the urokinase plasminogen activator receptor (uPAR). This receptor has been linked to the enhanced invasiveness of certain tumor cells. Insertion of an antisense RNA expression plasmid into the HEp3 human epidermoid carcinoma cells resulted in significantly reduced invasiveness of the transfected cells [69]. Moreover, the uPAR antisense RNA altered the phenotype of the Hep3 tumor cells from tumorigenic to dormant (determined by inoculation into the chorioallantoic membranes of chick embryos).

The invasive potential of human melanoma cells has also been suppressed using antisense RNA. In one study [70], an expression plasmid was used to synthesize RNA antisense to the secreted glycoprotein SPARC (secreted protein, acidic and rich in cysteine) in human melanoma cells. This treatment resulted in a marked decrease in the in vitro adhesive and invasive capacities of the melanoma cells and completely abolished their tumorigenicity in vivo. These results suggested that SPARC, which is expressed not only by melanomas but also by other types of tumors, might be a suitable target for antisense RNA treatment in order to block the invasion of tumors.

The hybrid bcr/abl gene product of the Philadelphia chromosome may also serve as a target for antisense inhibition because it is expressed as the result of an abnormal gene rearrangement (resulting in the formation of the Philadelphia chromosome), which occurs exclusively in chronic myeloid leukemia cells. It is not surprising, therefore, that inhibiting the expression of the abnormal gene product from the Philadelphia chromosome with antisense oligodeoxynucleotides has effectively blocked the proliferation of leukemia cells [71, 72]. In a related study [73], the expression of the bcr/abl gene product, protein P210 with a deregulated tyrosine kinase activity, was targeted using antisense RNA. For this purpose the antisense RNA was delivered and produced in human erythroleukemic K562 cells by means of a retroviral vector. This treatment resulted in an almost complete inhibition of the expression of P210 by the antisense transfected leukemic cells and in an almost twofold increase in their doubling time. This study suggested that such an antisense technique could be used for selective suppression of leukemic hematopoiesis.

Another protein involved in leukemic hematopoiesis, which has been shown to be a target for inhibition of antisense RNA, is phosphoprotein p18. Phosphoprotein p18 is expressed by leukemic cells from different lineages and plays an important role in cell cycle progression. Antisense RNA inhibition of p18 expression in leukemic K562 cells resulted in growth arrest at a lower saturation density, loss of serum independence and loss of anchorage-independent growth in vitro [74]. In addition, inhibition of the expression of p18 resulted in a marked inhibition of the tumorigenicity of leukemic cells in vivo in the severe combined immune deficiency mouse model. These studies demonstrated that the phosphoprotein p18 may also be a suitable target for antileukemic interventions.

The cyclins, which are important molecular components of the cell cycle and, therefore, are closely linked to cellular transformation and tumorigenesis, may also serve as targets for inhibition using antisense RNA. The cyclin G1 (CYCG1) is considered to be a protooncogene that is frequently overexpressed by human osteosarcoma cells. The expression of cyclin G1 in human MG-63 osteogenic sarcoma cells was reduced using a retroviral vector producing antisense RNA [75]. This treatment induced marked cytostatic and cytocidal effects in the antisense-transfected cells, suggesting that this might offer a potential gene therapeutic approach in the clinical management of osteogenic sarcoma. This idea was further supported by studies in which the same antisense cyclin G1 retroviral vector was delivered directly into rapidly growing subcutaneous osteogenic sarcoma-derived tumors in nude mice [76]. The antisense treatment inhibited the growth of the tumors, as demonstrated by decreased mitotic indices and increased stroma formation within the residual tumors. Furthermore, analysis of the cell cycle kinetics showed that there was a decrease in the number of cells in the S and G2/M phases of the cell cycle, concomitant with an accumulation of cells in the G1 phase.

Other interesting targets for antisense RNA inhibition are proteins linked to the resistance to chemotherapeutic agents that develops in tumor cells, as the development of drug resistance is a frequently encountered problem associated with the use of existing anticancer chemotherapy [77, 78]. For example, chloroethylnitrosourea (ACNU) is the drug of choice for chemotherapy of human malignant brain tumors. However, the cytocidal effect of ACNU is abrogated through repair of the ACNU-induced lesions in DNA by the enzyme O^6-methylguanine-deoxyribonucleic acid methyltransferase (MGMT). This enzyme is found in several different types of solid tumors, among which are brain tumors. An antisense RNA expression plasmid directed to the enzyme MGMT was transfected into a clone of C6 glioma cells, C6AR, which were resistant to ACNU [79]. The antisense treatment resulted in a significant repression of the resistance of C6AR tumor cells to ACNU, suggesting that this might be a useful strategy for overcoming ACNU resistance in the treatment of gliomas.

It should be emphasized that the studies outlined above are only a few examples from the many different types of proteins that may be targeted with antisense RNA in order to limit and inhibit tumor cell growth. Indeed, whenever the nucleic acid sequence encoding a new protein linked to tumor cell progression is discovered, this protein could serve as a target for antisense RNA. This antisense RNA may help to uncover the function of this protein and possibly to develop useful antisense RNA therapeutic agents.

Calmodulin antisense RNA

Another protein which is essential for cell proliferation and which has been linked to cell differentiation is calmodulin. Calmodulin has been found to be overexpressed by certain types of tumor cells, such as gliomas [80], breast cancer [81] and leukemic cells [82]. Calmodulin is a ubiquitous, highly conserved protein with diverse functions. In humans and rats it is encoded by three genes that collectively give rise to five transcripts, all of which encode a protein with an identical amino acid sequence [83]. The levels of the individual calmodulin transcripts arising from the three calmodulin genes vary during the differentiation of PC12 cells [84, 85]. Further, reducing the levels of calmodulin in PC12 cells using calmodulin antisense RNA produced by a mammalian expression plasmid reduced the rates of cell proliferation and also induced cell differentiation [30]. Since PC12 cells have been originally derived from the adrenal medullary tumor pheochromocytoma, these studies suggested that it would be possible to block cell proliferation and induce differentiation of tumor cells by inhibiting the expression of calmodulin. One problem with this approach, however, is the fact that although calmodulin is overexpressed by certain tumor cell types, it is expressed, albeit at lower levels, in all normal cells as well. We reasoned that a possible solution to this problem might be achieved by targeting only the transcripts from the most abundantly expressed (dominant) calmodulin genes found in the tumor cells. For example, the transcripts from calmodulin genes I and II are dominant in PC12 cells, whereas the transcripts from calmodulin gene III are found at very low levels in these cells [84, 86]. In contrast, in certain lymphoblastoid cells, the transcripts from calmodulin gene III are dominant, whereas in normal quiescent lymphocytes, the transcripts from calmodulin gene III are not elevated [82]. To determine whether we can selectively inhibit the dominant forms of the calmodulin transcripts using antisense compounds, and in this way selectively reduce the proliferation of cells overexpressing these transcripts, we first employed antisense oligodeoxynucleotides [27]. Selective treatment of PC12 cells with antisense oligodeoxynucleotides targeted to the dominant transcripts from calmodulin genes I or II inhibited the proliferation of PC12 cells and altered the course of their differentiation. In contrast, treatment of PC12 cells with antisense oligodeoxynucleotides to the transcripts from calmodulin gene III, which are found at low levels, did not inhibit their proliferation nor alter their course of differentiation. These results suggested that it might be possible to inhibit the proliferation of tumor cells and influence their differentiation by specifically reducing the expression of the elevated calmodulin transcripts using antisense compounds.

In an attempt to achieve long-term inhibition of the transcripts from calmodulin gene I in PC12 cells, we developed an RNA expression vector which contains a DNA sequence that encodes an antisense RNA targeted to the two transcripts derived from calmodulin gene I (fig. 1). The antisense RNA produced by this vector is 115 nucleotides long and is complementary to the entire 5′ untranslated region of both the 1.7- and 4.1-kb transcripts derived from calmodulin gene I. This portion of the calmodulin message was chosen as a target because the 5′ untranslated region of each individual calmodulin gene is unique [83]. PCR-cloning methodology was employed to obtain a 115-bp sequence spanning the 5′ untranslated region and the initiation codon [nucleotides -110 to $+5$ of the complementary DNA (cDNA)] of rat calmodulin gene I. This PCR product was inserted in the antisense orientation in the expression vector pCR3 (Invitrogen). This antisense vector (CaM I-5′ AS) would be expected to target both transcripts from calmodulin gene I in PC12 cells which are expressed at high levels [84, 86].

To examine the biological effects of this vector, the CaM I-5′ AS or a control 'empty' pCR3 vector was transfected into PC12 cells using lipofectin. As anticipated from the experiments using antisense oligodeoxynucleotides, PC12 cells transfected with the antisense vector evidenced slower rates of proliferation in comparison with the cells transfected with the empty vector (fig. 2). These studies indicated that it may be feasible to reduce the rates of cell proliferation by inhibiting the expression of calmodulin using specifically designed antisense RNA expression vectors.

Several additional strategies could be applied in order to reduce the proliferation of only tumor cells while sparing the normal cells which do not overexpress calmodulin. As suggested earlier, one way would be to selectively inhibit the expression of only those calmodulin transcripts which are overexpressed in a particular tumor cell type. For example, studies done in our laboratory showed that the calmodulin transcripts expressed by certain human breast cancer cell lines are different from the calmodulin transcripts expressed by normal human breast cells (fig. 3). Using Northern blotting, we characterized the pattern of expression of the calmodulin transcripts in RNA extracted from normal human breast tissue and from two different human breast adenocarcinoma cell lines, MCF7 and SkBr3. We found that the RNA from normal human breast tissue displayed two calmodulin transcripts: one of these corresponded in size to the 4.1-kb transcript from calmodulin gene I, and the other to the 1.4-kb transcript from calmodulin gene II. The RNA from the breast adenocarcinoma cell lines MCF7 and SkBr3 also displayed

the 4.1-kb transcript from calmodulin gene I and relatively small quantities of the 1.4-kb transcript from calmodulin gene II. However, in addition to these two transcripts, the RNA from both breast cancer cell lines showed evidence for a 1.7-kb transcript from calmodulin gene I. Since we did not detect this transcript in the normal human breast tissue, and since inhibiting the expression of the transcripts from calmodulin gene I has been shown to reduce cellular proliferation [27], it might be possible to selectively inhibit the proliferation of the breast cancer cells using an antisense RNA directed specifically to the 1.7-kb transcript from calmodulin gene I.

A further level of selectivity could be achieved by constructing a molecular conjugate vector, in which the expression vector is complexed to an antibody that recognizes a cell surface antigen expressed by the targeted tumor cell type. In addition, the expression vector may be constructed so that the calmodulin antisense RNA sequence is expressed from a cell-specific promoter. For example, if the calmodulin antisense RNA is intended to target glial tumors, the glial fibril-
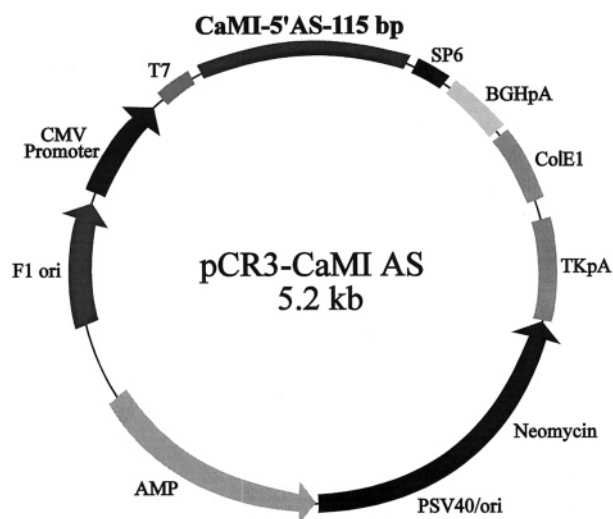

Figure 1. Construction of a calmodulin gene I antisense RNA vector. A vector producing antisense RNA to the 1.7- and 4.1-kb transcripts from calmodulin gene I was constructed by PCR cloning using the plasmid containing the cDNA from calmodulin gene I (pRCMI) and the expression plasmid pCR3 (Invitrogen). Shown are the elements of the antisense RNA expression vector (CaMI-5′ AS), which has a molecular size of 5.2 kb: cytomegalovirus immediate early promoter (PCMV), followed by a T7 promoter, calmodulin gene I cDNA sequence of 115 bp containing the entire 5′ untranslated region and the initiation codon, SP6 promoter, bovine growth hormone polyadenylation signal (BGHpA), ColE1 origin of replication, thymidine kinase polyadenylation signal (TkpA), neomycin resistance gene (Neomycin), SV40 origin of replication (Psv40/ori), ampicillin resistance gene (AMP) and F1 origin (F1 ori).
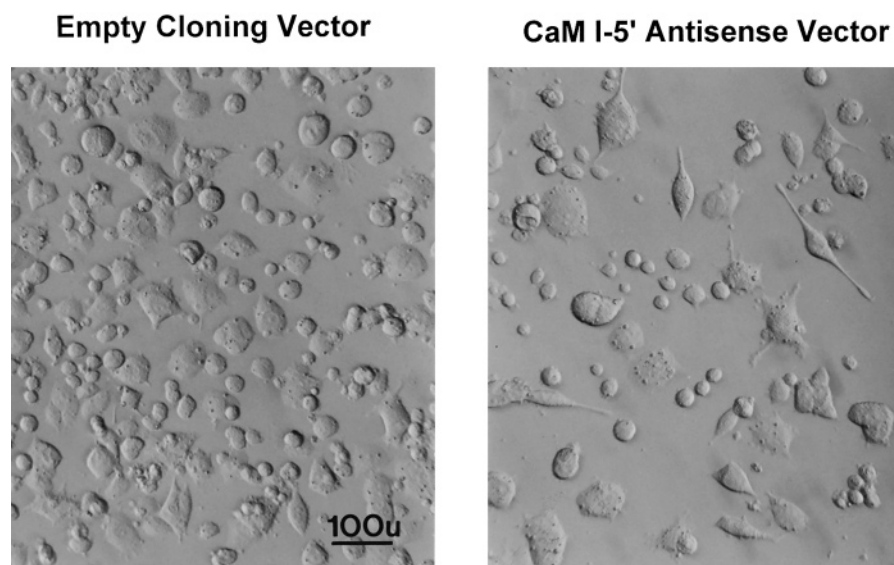
Empty Cloning Vector   CaM I-5' Antisense Vector

Figure 2. Transfecting PC12 cells with calmodulin gene I antisense RNA vector inhibits cell proliferation. The antisense RNA vector CaMI-5′ AS or an empty vector pCR3 control was transfected into PC12 cells using lipofectin as described earlier [30]. The photomicrographs show typical morphology and cell density of PC12 cells grown in 100-mm plates and transfected with empty cloning vector (left), or calmodulin gene I antisense vector, CaMI-5′ AS (right). As may be seen, the cells transfected with CaMI-5′ AS evidenced slower proliferation and lower cell densities in comparison with the cells transfected with the empty vector. More cells transfected with CaMI-5′ AS evidenced neurite outgrowth in comparison with the cells transfected with the empty vector. These results suggest that inhibiting the calmodulin gene I transcripts, which are dominant in PC12 cells, inhibits their proliferation and may also induce differentiation.

lary acidic protein promoter could be used [87, 88]. In this way, each expression vector could be tailored in such a way as to deliver and express the antisense RNA only in the tumor cells and to inhibit only the dominant calmodulin transcripts found in these cells, thereby reducing the growth of the target tumor cells while sparing normal cells.

Infectious diseases

Investigations have been carried out to evaluate the potential utility of vectors expressing antisense RNA for gene therapy of certain infectious diseases. Several antisense RNA expression vectors have been developed which have demonstrated in vitro and in vivo efficacy in protecting cells against human immunodeficiency virus (HIV) infection. The acquired immune deficiency syndrome (AIDS) virus would be an especially suitable target for antisense RNA because of the rapid characterization of viral and host molecular structures which have been linked to the pathogenesis of HIV-1 infection and AIDS. A series of studies have been focused on the intracellular inhibition of HIV-1 replication through the use of antisense RNA in order to interfere with the activity of two key HIV-1 regulatory proteins, Tat and Rev, which are powerful trans-activators of HIV-1 viral gene expression. In a series of studies by Morgan and

colleagues, retroviral vectors have been employed to produce antisense RNA targeted at the HIV-1 TAR element, which is present within the untranslated leader sequence of all HIV-1 transcripts, including the HIV RNA genome, and is the region to which Tat binds to activate transcription [89, 90]. In studies using transient and stable transfection assays in vitro, it was shown that a retroviral vector expressing antisense HIV-1 TAR inhibited Tat-mediated transactivation of a chloramphenicol acetyltransferase (CAT) reporter plasmid with the HIV-1 LTR genes [89]. Subsequently, a clinical trial protocol for AIDS was proposed based on this study, in which a retroviral vector would be used to deliver antisense TAR genes to syngeneic lymphocytes obtained from HIV-seronegative identical twins [90]. The potential efficacy of these genetically engineered lymphocytes on the functional immune status would then be evaluated following adoptive transfer in HIV-infected twin recipients [91].

In another study, retroviral vectors expressing HIV-1 tat or rev antisense RNA were evaluated for their ability to protect Jurkat cells from HIV-1 infection [92]. This study showed that HIV-1 tat or rev antisense RNA can protect the cells after challenge with HIV-1, with the degree of protection being determined primarily by the type of expression system utilized. They found that

antisense RNA expression driven by a transfer RNA (tRNA) promoter in the context of a double-copy vector conferred better long-term protection against HIV-1 infection than that driven by HIV LTR or MLV LTR promoters. These investigators properly underscore the need to optimize the structure of the expression vectors in order to develop useful antisense compounds for gene therapy.

In addition to the development of antisense RNA expression vectors, antisense RNA gene therapy of HIV has been evaluated as a combination therapy with cur-
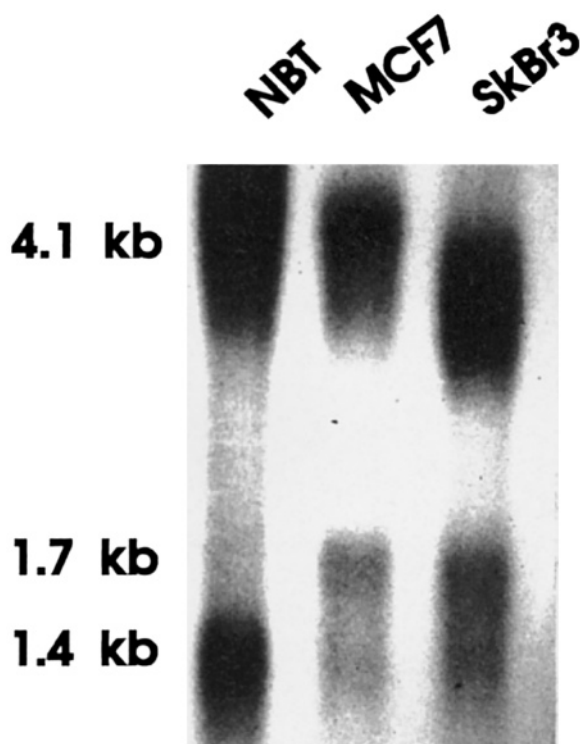

Figure 3. Relative abundance of the individual calmodulin transcripts in normal and malignant human breast cancer cells. The human breast adenocarcinoma cell lines MCF7 and SkBr3 were purchased from the American Type Culture Collection and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, insulin (10 μg/ml) and an antibiotic-antimycotic reagent. Total cellular RNA was isolated from the cultured cells using the Trireagent method as described earlier [30]. The total cellular RNA from normal human breast tissue was obtained from Clonetech. To determine the expression of the individual calmodulin transcripts, the RNA samples were subjected to Northern blot performed with a ^{32}P-labeled calmodulin riboprobe according to a procedure described by Bai and Weiss [84]. The results showed that there was a distinct pattern of calmodulin transcripts expressed in the RNA from the breast cancer cell lines, MCF7 and SkBr3, which was different from the pattern of the calmodulin transcripts expressed in the RNA from the normal human breast tissue (NBT). More specifically, the breast cancer cells contained a calmodulin transcript of approximate size of 1.7 kb that was not present in the normal breast cells. These results suggest that it may be possible to selectively block the proliferation of the breast cancer cells, while sparing the normal cells, by using antisense RNA targeted to the 1.7-kb transcript from calmodulin gene I.

rently existing antiviral medications (e.g. reverse transcriptase and protease inhibitors) [93]. It was reported that the combination of Gag antisense RNA with clinically relevant reverse transcriptase inhibitors (e.g. azidothymidine and dideoxycytosine) or protease inhibitors (inclinavir) was 10-fold more effective at inhibiting HIV replication than the single antiviral regimen alone. More important, Gag antisense RNA showed antiviral efficacy against reverse transcriptase inhibitor-resistant HIV-1 isolates [93].

Chronic infection with the hepatitis B virus (HBV) is another candidate condition for gene therapy using antisense RNA. Vector expression systems producing antisense RNA have been transfected into hepatocytes in vitro and have demonstrated the ability to generate efficient antiviral effects during chronic HBV infection [94]. These findings may be of significant therapeutic value considering the serious consequences from HBV infection, such as fulminant hepatitis, chronic hepatitis, cirrhosis and hepatocellular carcinoma.

Cardiology

Promising results have been obtained in studies using vectors expressing antisense RNA in order to control cardiovascular diseases. Some studies have targeted components of the renin-angiotensin system with antisense RNA in order to lower blood pressure. One study performed in vitro established the possibility of using vectors producing antisense RNA in order to inhibit expression of the mRNA encoding angiotensinogen [95]. Another series of studies demonstrated the possibility of producing prolonged reduction of high blood pressure in vivo by using an adeno-associated viral vector producing antisense RNA to the angiotensin type 1 receptor [96, 97]. In initial studies, the efficacy of a recombinant adeno-associated viral cassette containing the angiotensin receptor antisense RNA gene was established in vitro by their finding that stably transfecting the vectors into neuroblastoma NG108−15 cells resulted in significant reductions in the levels of angiotensin type 1 receptors expressed by these cells. Subsequently, this angiotensin receptor antisense RNA expression cassette was administered as a single injection into the hypothalamus or into the lateral ventricles of adult spontaneously hypertensive rats [97]. This treatment produced a significant decrease in blood pressure for up to 9 weeks after a single injection.

The approaches described above would have the advantage over existing pharmacological agents for treating hypertension in that the antisense-generating agents would have to be administered less frequently. In addition, the antisense RNA strategy would not be expected to cause the pulmonary side effects caused by some currently used drugs that act by interfering with the renin-angiotensin system. For example, the angiotensin-converting enzyme (ACE) inhibitors, which are very

effective at lowering high blood pressure in many patients, produce their antihypertensive action by blocking the active site of the angiotensin-converting enzyme and, thus, the conversion of angiotensin I to angiotensin II. However, the ACE inhibitors also inhibit the degradation of other peptides, such as bradykinin, substance P and enkephalins, which may be responsible for the cough and angioedema sometimes associated with their use. One possible problem with an antisense RNA approach, however, would be the need to develop effective systemic routes of delivery of the antisense RNA vectors. In this regard, initial studies have already demonstrated a reduction in blood pressure for at least 5 weeks after a single intracardiac injection of an adeno-associated virus expressing an angiotensin type 1 receptor antisense RNA into young spontaneously hypertensive rats [97].

The possibility of using antisense RNA in order to attenuate the actions of angiotensin and, thereby, to reduce high blood pressure was determined in another set of studies [98] in which an adenoviral vector was used to deliver antisense RNA to target cells expressing the angiotensin type 1 receptor (AT1-R), that is hypothalamic brain stem neurons in culture and cultures of vascular smooth muscle cells. The delivery of the AT1-R antisense RNA vector into the cells resulted in the synthesis of AT1-R antisense RNA in cells and a concomitant inhibition of the actions of angiotensin II mediated by AT1-R. Thus, the decreased responsiveness to angiotensin II of antisense-treated vascular smooth muscle cells was evidenced by a significant decrease in the incorporation of [^{3}H]thymidine. Decreased responsiveness to angiotensin II of antisense-treated neurons was evidenced by a decreased uptake of norepinephrine in these neurons, which is ordinarily stimulated by angiotensin II. These effects appeared to be specific since they were not produced by the control adenoviral vector. Thus, these studies further confirmed the possibility of using antisense RNA to the angiotensin type 1 receptor in order to treat hypertension.

In another interesting study, antisense basic fibroblast growth factor gene transfer was employed to reduce neointimal thickening after arterial injury [99]. In this study, the carotid artery of rats was first subjected to injuries using a balloon catheter, and then the rats were infected with an adenoviral vector-encoding antisense RNA to basic fibroblast growth factor. The effective reduction in neointimal thickening by the antisense RNA suggested that this might be a feasible approach to limiting the restenosis after angioplasty.

Neurology and psychiatry

A wide variety of antisense oligodeoxynucleotides have been used successfully to block in vivo the expression of functionally important proteins in the nervous system, such as receptors for neurotransmitters and neuropeptides, some of which may be involved in the pathogenesis of certain neurologic or psychiatric disorders (reviewed in [21, 23, 100, 101]). These targets included neurotransmitter receptors, for example the muscarinic [102], D1 dopamine [103], D2 dopamine [104–108], D3 dopamine [25, 109, 110], D5 dopamine [111], N-methyl-D-aspartate [112, 113] and serotonin [114] receptors; peptide receptors, for example the angiotensin type 1 [115–118], cholecystokinin [119], neuropeptide Y [120], substance P [121] and vasopressin [122] receptors; various subtypes of opioid receptors [123–126]; steroid receptors, such as the estrogen [127] and progesterone [128, 129] receptors, and many other nonreceptor proteins and immediate early genes. (For a more detailed review of the targets in neuronal cells which have been inhibited using antisense oligodeoxynucleotides see [21].) Any of these proteins could serve as potential targets for antisense inhibition using vectors expressing the appropriate antisense RNA. Our laboratory has chosen such an approach to study the feasibility of producing specific, long-term inhibition of the synthesis of the different subtypes of the dopamine receptor found in the central nervous system.

D2 dopamine receptor antisense RNA

Multiple subtypes of dopamine receptors. The dopamine receptor family consists of two subgroups: one subgroup contains the D1 and D5 subtype and the other the D2, D4 and D5 subtypes. These five dopamine receptor subtypes, which are widely but unequally distributed in the nervous system [130–132], are thought to be involved in numerous and diverse disorders of the central nervous system, including psychiatric disorders such as schizophrenia [133–135], addiction to substances of abuse such as cocaine [136, 137] and alcohol [138], diverse movement disorders such as Parkinson's disease [139, 140], Huntington's chorea [140, 141] and tardive dyskinesia [142, 143], and endocrine disorders such as hyperprolactinemia [144, 145]. Accordingly, selective long-term inhibition of the synthesis of one of the dopamine receptor subtypes, particularly if the reduction of dopamine receptors can be localized to discrete brain regions, offers the potential of providing a novel and effective means of treating certain disorders associated with dopaminergic hyperactivity.

We have chosen the D2 dopamine receptor subtype as a model to target with antisense compounds for several reasons. The ability of the currently used traditional antipsychotic drugs to block the D2-like dopamine receptors has suggested that these receptors may be involved in schizophrenia [135], and the upregulation of these receptors produced by long-term treatment with antipsychotic drugs often leads to the chronic debilitating disorder termed tardive dyskinesia [142]. There are suitable in vivo models to study the D2 dopamine

receptor, such as the 6-hydroxydopamine model of dopaminergic supersensitivity, and well-characterized behaviors to assess the function of both the D2 and D1 dopamine receptors. There are also ready means of assessing the levels of these receptor subtypes in tissue using conventional radioligand techniques. Therefore, we would be in a position to measure the specificity with which the antisense RNA strategy exerts its effects at the molecular, biochemical and behavioral levels.

Construction of D2 antisense RNA expression vectors. Our laboratory has developed two plasmid vectors expressing antisense RNA to the D2 dopamine receptor, one for in vitro use and one for in vivo use [29, 32, 146]. The expression vector used for the in vivo studies was plasmid pCR3 (Invitrogen), that is the same vector used for cloning the calmodulin gene I antisense RNA, which was described in the earlier section on calmodulin antisense RNA and in figure 1. The expression vector used for the in vitro studies was plasmid pCEP4 from Invitrogen, which was similar to plasmid pCR3. Both of these vectors contained a CMV promoter followed by an identical cDNA sequence antisense to a portion of the D2 dopamine receptor mRNA, and both of them contained the genes conferring resistance to ampicillin and geneticin. However, the vector used for the in vitro studies was larger in size (10,722 bp) because it contained additional elements for extrachromosomal replication and for resistance to hygromycin. These features made this plasmid vector more suitable for the selection of stable transfectants because the cells that we used in our in vitro studies (see below) had already been rendered resistant to geneticin. Unlike the D2 antisense RNA vector used in the in vitro studies, the vector for the in vivo studies was smaller (5422 bp), which would presumably offer the advantage of more easily penetrating into the brain cells in vivo.

To construct the D2 antisense vector that was used in vivo, PCR cloning was employed. A portion of the D2 dopamine receptor cDNA which spans the third intracellular loop of the D2 dopamine receptor was amplified by PCR and then inserted in the antisense orientation relative to the CMV promoter in pCR3. This cDNA sequence, which gives rise to a 337-bp D2 dopamine receptor antisense RNA, was selected because it shares the least homology with the remaining dopamine receptor subtypes [146]. To construct the D2 antisense vector for in vitro use, the D2 antisense sequence was excised from the vector used in the in vivo studies, using appropriate restriction enzymes, and ligated into plasmid vector pCEP4. Using these D2 antisense vectors, we performed a series of in vitro studies, in which we provided proof of the mechanism of antisense action, and a series of in vivo studies, in which we correlated biochemical, molecular biological and behavioral effects of the D2 antisense vector. To enhance the

cellular uptake of the plasmid vectors, we used two different cationic lipids, which were chosen based on preliminary studies on their effectiveness and relative lack of toxicity; that is lipofectin was used for the in vitro studies, and 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) was used for the in vivo studies.

In vitro studies providing proof that the D2 antisense vector acts through an antisense mechanism. Several conditions should be met before one can conclude that an antisense RNA expression vector is acting through a true antisense mechanism. The most important of these conditions is the demonstration that the antisense vector (i) enters the cells and persists for a sufficient period of time to produce a biological effect; (ii) generates antisense RNA inside the cells; (iii) reduces the level of the target protein; (iv) produces appropriate and specific biological effects; and (v) shows a positive and, if possible, a temporal correlation between the formation of antisense RNA in cells and a reduction in the target protein. Some of these points, such as the biochemical and molecular correlates of the antisense RNA vectors, are most readily demonstrated in cultured cells because they express high levels of the targeted protein. Others, such as the functional and temporal correlates of the antisense RNA vectors, are best determined using an in vivo system. Accordingly, in the studies reported below, we will use as illustrations the particular systems that most appropriately demonstrate each of the criteria listed above. In initial studies aimed at proving the antisense mechanism of action of the D2 antisense vector described above, we used as an in vitro model human embryonic kidney (HEK) cells that had been stably transfected with the long isoform (D2L) of the D2 dopamine receptor gene, termed HEK 293$_{DL}$ cells. These cells were useful for determining and correlating certain molecular and biochemical effects of the D2 antisense vector because they provide a relatively homogeneous population of cells which can be easily grown to large quantities, because they can be readily transfected and because they have been previously found to stably express a high density of D2 dopamine receptors [147].

Because there are as yet no general rules for choosing the optimal site to target within a given transcript nor the optimal length of the antisense RNA, these parameters have to be established separately for each individual transcript. Accordingly, one aim of the in vitro studies was to determine whether the chosen D2 antisense RNA sequence could inhibit the expression of D2 dopamine receptors. Another aim was to determine whether the D2 antisense vector acts by a true antisense mechanism. In this section we will present studies providing biochemical and molecular evidence supporting a true antisense effect of the D2 antisense RNA. In later sections, we will describe in vivo studies which

provided additional evidence that the D2 antisense vector was acting through a true antisense mechanism.

In these in vitro studies we showed, using reverse transcriptase PCR (RT-PCR), that only the HEK 293$_{DL}$ cells that were transfected with the D2 antisense vector generated the predicted D2 antisense RNA; the original HEK cells, the HEK cells transfected in the D2 dopamine receptor gene (HEK 293$_{DL}$) and the HEK 293$_{DL}$ cells transfected with the empty vector did not. Further, we found that the D2 dopamine receptor mRNA was reduced to undetectable levels in HEK 293$_{DL}$ cells stably transfected with the D2 antisense vector. In contrast, using RT-PCR, D2 dopamine receptor mRNA was readily detected in the parental HEK 293$_{DL}$ cells and in the HEK 293$_{DL}$ cells transfected with the empty vector. The decrease in the D2 receptor mRNA in the D2 antisense vector-transfected cells was not likely due to nonspecific general effects of the transfection procedures on transcription since we did not detect any changes in the levels of the mRNA of the housekeeping gene β-actin. Additional biochemical evidence in support of the specific antisense effect of the D2 antisense vector in HEK 293$_{DL}$ cells was provided by data showing a marked decrease in the levels of D2 dopamine receptors. Thus, the density of D2 dopamine receptors in the cells transfected with the D2 antisense vector was significantly lower than the density of D2 dopamine receptors in cells transfected with the empty vector, as determined in a radioligand binding assay [32]. Taken together, these data suggest that it was the generation of D2 antisense RNA in HEK 293$_{DL}$ cells stably transfected with the D2 antisense vector that caused the reduction in the levels of D2 dopamine receptors.

Having shown that the chosen D2 antisense RNA sequence could inhibit the expression of D2 dopamine receptors, and having provided proof of a true antisense mechanism for this D2 antisense-generating vector in vitro, we then turned our attention to the in vivo effects of the D2 antisense vector.

Long-term presence of the D2 antisense RNA expression vector in brain. Previous studies with D2 antisense oligodeoxynucleotides showed that it is possible to selectively inhibit D2 dopamine receptor-mediated responses after either intracerebroventricular [31] or intrastriatal [106] injections of the oligodeoxynucleotide into a discrete brain area, the corpus striatum. However, these effects were short lasting, and the inhibited behaviors returned to normal after only 2 to 3 days. Therefore, the antisense oligodeoxynucleotides had to be repeatedly administered in order to have prolonged reductions in dopaminergic functions. As a long duration of action may be one of the major advantages that an antisense RNA technique might have over the use of antisense oligodeoxynucleotides, studies were designed to determine whether the D2 antisense RNA plasmid

vector would persist for a prolonged period of time in brain after a single administration. Initially, we used PCR techniques to determine for how long the D2 antisense plasmid vector persisted into the corpus striatum of mice after a single administration. Then, we evaluated the functional effects of the D2 antisense vector by measuring certain D2 dopamine receptor-mediated behaviors. In these studies the D2 antisense plasmid, complexed with DOTAP, was injected directly into the corpora striata, and a series of biochemical and behavioral studies was performed [32, 146, 148]. In one, the mice were killed at various time points after the injection, ranging from 5 min to 96 days, and the presence of the D2 antisense plasmid was characterized by solution-phase PCR with primers specific for the SP6 and T7 promoters, which flank the D2 antisense sequence in the vector polylinker (fig. 4). A PCR product, corresponding to the size of the D2 antisense insert (489 bp), was found in the injected striatum at 5 min and at 3, 6, 12 and 24 days after the injection (fig. 4). This PCR product was not detected in the injected striatum at 48 or 96 days after the injection or in the uninjected striatum at any time point examined.

Long-term in vivo inhibition of D2 dopamine receptor-mediated behaviors produced by the D2 antisense vector. We used two different model systems to determine the effects of the D2 antisense vector in vivo. One model employed mice with normal dopamine functions, and the other model used mice which had supersensitive dopaminergic activity. Supersensitive dopaminergic function was induced by injecting mice into the corpus striatum with the dopaminergic neurotoxin 6-hydroxy dopamine. This treatment causes an increased level of D2 dopamine receptors in the injected striatum [105, 149] and a specific turning behavior contralateral to the 6-hydroxydopamine lesion in mice given challenge injections with the D2 dopamine receptor agonist quinpirole [150, 151]. The results showed that treatment of mice with single injections of the D2 antisense vector into the lesioned striatum blocked the rotational response to challenge injections with quinpirole. Importantly, a single injection of the D2 antisense vector produced a D2 dopamine receptor blockade that lasted about 1 month [146, 148].

To determine the effects of the D2 antisense vector in normal mice, two different behaviors were studied, both of which are mediated by D2 dopamine receptors. One was a cataleptic behavior and the other was a climbing behavior produced in response to challenge injections with the D2 dopamine receptor agonist apomorphine. Both of these behaviors are manifested when the activity of D2 dopamine receptors in the corpora striata is inhibited. In these studies, the D2 antisense vector was injected bilaterally into the corpora striata, and these two behaviors were measured at various time points
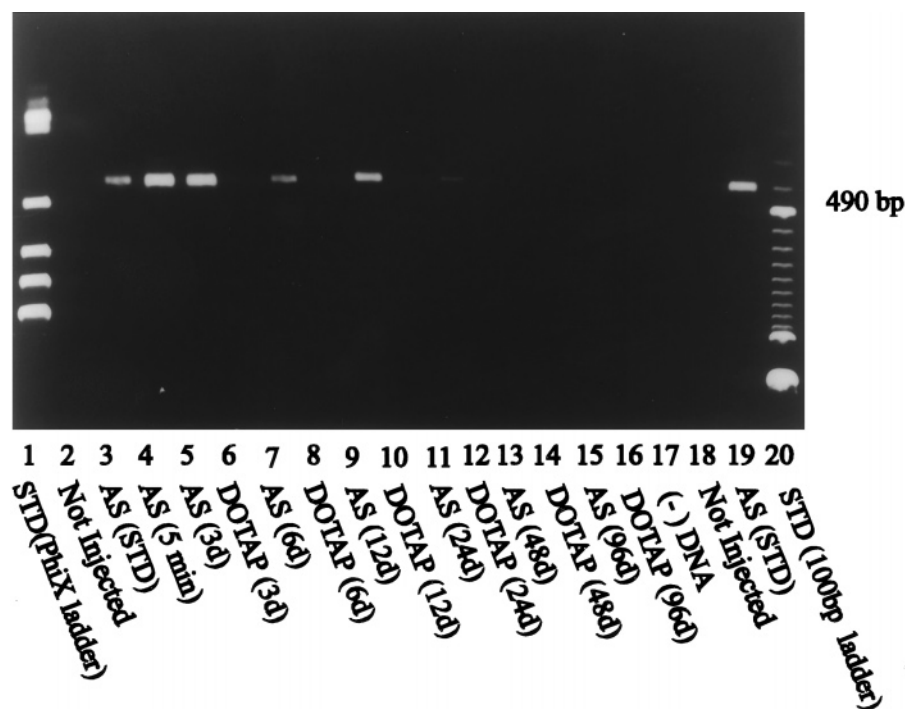
Figure 4. Long-term presence of a D2 antisense RNA vector in mouse striatum after a single administration. Mice were administered a single injection of a D2 antisense RNA plasmid vector (AS) complexed with DOTAP (25 μg of DNA and 10 μg of DOTAP) into one corpus striatum. The control mice received DOTAP alone. At different times thereafter, DNA was extracted from the injected striata using proteinase K/SDS treatment and was subjected to PCR amplification with T7 (5′-TAA TAC GAC TCA CTA TAG GG-3′) and SP6 (5′-GAT TTA GGT GACACT ATA G-3′) primers, which bound to sites flanking the vector polylinker, as described earlier [146]. A D2 antisense plasmid vector purified from *E. coli* using Qiagen columns was used as a standard. The PCR product of this vector served as the positive control [AS(STD)]. The PCR products were separated on a 2.5% agarose gel and visualized by staining with ethidium bromide. The size of the bands corresponding to the PCR product from the D2 antisense vector (490 bp) was approximated by comparison with the migration of the two DNA size markers (PhiX 174 digested with *Hae*III from Promega (STD/Phi X ladder) and a 100-bp DNA ladder from GIBCO (STD/100-bp ladder). The identity of each sample is indicated below the figure as follows: Not Injected, striata from noninjected mice; AS (5 min), AS (3d), (6d), (12d), (24d), (48d) and (96d), striata from mice injected with the D2 antisense vector and killed at 5 min, and 3, 6, 12, 24, 48 and 96 days, respectively, after the injection; DOTAP (3d), (6d), (12d), (24d), (48d) and (96d), striata from control mice injected with DOTAP and killed at 3, 6, 12, 24, 48 and 96 days after the injection; (-) DNA, PCR reaction performed in the absence of the DNA template. The results showed that the D2 antisense vector was present in the injected striatum for up to 24 days after a single administration. (Taken from [32].)

after the injection of the vector. The results showed that single bilateral intrastriatal injections of the D2 antisense vector induced catalepsy as early as 3 days after the injection, and this behavior lasted for about 30 days (fig. 5A) which corresponded to the length of time during which the plasmid DNA was found to be present in the striatum (see fig. 4). To determine whether it was possible to extend the inhibitory effect of the D2 antisense vector on D2 dopamine receptors, a second bilateral intrastriatal injection of the vector was administered at a time point when the initial effect of the D2 antisense vector had disappeared. In this case the second injection of the D2 antisense vector was administered at 40 days after the first injection. Figure 5A shows that the second injection of the vector also caused a significant cataleptic response, which was statistically significant for an additional 30 days.

Similarly, treatment with the D2 antisense vector inhibited apomorphine-induced climbing behavior. Again, the inhibition of this D2-mediated response was seen as early as 3 days after the injection and lasted for about 30 days. The second injection of the D2 antisense vector, given at a time when the behavioral response had returned to normal, blocked apomorphine-induced climbing for an additional month (fig. 5B). The specificity of the observed effects of the D2 antisense vector was ascertained by performing parallel studies with a control, empty vector; the empty vector failed to produce any significant effects.

These results show that single injections of the D2 antisense vector block the functions of the D2 dopamine receptors for a far greater length of time than did comparable injections of a D2 antisense oligodeoxynucleotide and demonstrated further that

prolonged reductions in dopaminergic activity can be achieved with the D2 antisense vector without resulting
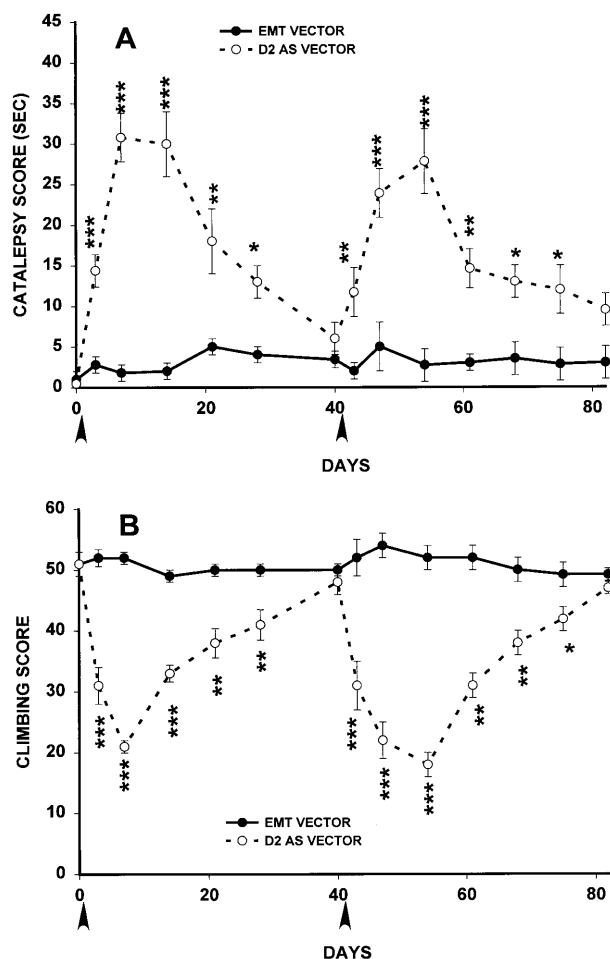

Figure 5. Long-term effects of D2 antisense RNA vector on D2 dopamine receptor-mediated behaviors. The D2 antisense RNA vector or empty vector (25 μg each), complexed with DOTAP (10 μg), was injected bilaterally into each corpus striatum of mice (first arrowhead), and catalepsy (*A*) or climbing induced by challenge injections of apomorphine (*B*) was determined at various times thereafter. At 40 days after the initial injections of the vector, a time at which catalepsy disappeared and the inhibition of apomorphine-induced climbing recovered, the mice were given a second bilateral intrastriatal injection of the D2 antisense vector or empty vector complexed with DOTAP (second arrowhead). Catalepsy and apomorphine-induced climbing were determined thereafter at various times for an additional 40 days. Each point represents the mean value obtained from 8–15 mice. Vertical brackets indicate the SE. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with values from mice treated with empty vector. The graphs show that a single injection of the D2 antisense vector induced a cataleptic response and inhibited the climbing response induced by apomorphine. These behaviors, which result from an inhibition of D2 dopamine receptors, lasted about 30 days. Similarly, a second injection of the D2 antisense vector produced effects (also lasting about 30 days) that are consistent with the inhibition of D2 dopamine receptor-mediated behaviors. The onset and duration of the effects of the second injection of the D2 antisense vector were similar to those seen after the first injection of the D2 antisense vector.

in behavioral tolerance to this treatment, a result which may have important clinical consequences.

The D2 antisense RNA vector induces long-term inhibition of D2 receptor functions in vivo without inducing dopaminergic supersensitivity. A general principle in biology, originally derived from early studies on the adrenergic receptor-adenylate cyclase system of the pineal gland [152–154] and subsequently confirmed in many other receptor systems [155, 156], holds that the density and function of neuroreceptors (and perhaps other regulatory proteins) are inversely related to the degree to which they have been activated. That is, long-term inhibition of the activity of a receptor causes a cascade of intracellular events leading to upregulation of the inhibited protein. (The exact mechanism of this receptor upregulation is unknown but may involve changes in the levels of cyclic adenosine 3′,5′-monophosphate (cyclic AMP) which, in turn, may alter the transcription of genes that have cyclic AMP regulatory elements (CRE) located in their promoters, or may involve other as yet unknown mechanisms [25, 157]. Such a process could lead to tolerance to the agent that was used to inhibit the receptor and, in some cases, to unexpected serious side reactions.

In the dopaminergic system, this phenomenon has been used to explain not only the tolerance that may develop to certain dopamine receptor antagonists but also the abnormal motor activity often associated with their long-term use. Long-term treatment with many antipsychotic drugs, which produce their therapeutic actions by inhibiting the activity of D2 dopamine receptors by blocking the interactions of the neurotransmitter with their target receptor, causes an increase in the density of the very receptors they are designed to inhibit. One of the consequences of this upregulation of D2 dopamine receptors is an abnormal motor response termed tardive dyskinesia. We reasoned that, by using an antisense approach, the antisense agent would reduce the levels of functional receptors by reducing their rate of synthesis. By reducing the synthesis of receptors, perhaps the receptors would not be able to upregulate.

To test this hypothesis, we employed as a positive control a drug that has previously been shown to induce dopaminergic hyperactivity both at the receptor level (increased density of D2 dopamine receptors) and behavioral level (increased behavioral effects in response to challenge injections with dopamine receptor agonists). Such an agent is the widely used antipsychotic drug haloperidol. The behavioral model used to test this hypothesis is the climbing response induced in rodents by the dopamine receptor agonist apomorphine. Pretreatment with haloperidol, by blocking the D2 dopamine receptors, ordinarily antagonizes the increased

climbing induced by dopamine receptor agonists. How-ever, prolonged treatment with the dopamine receptor
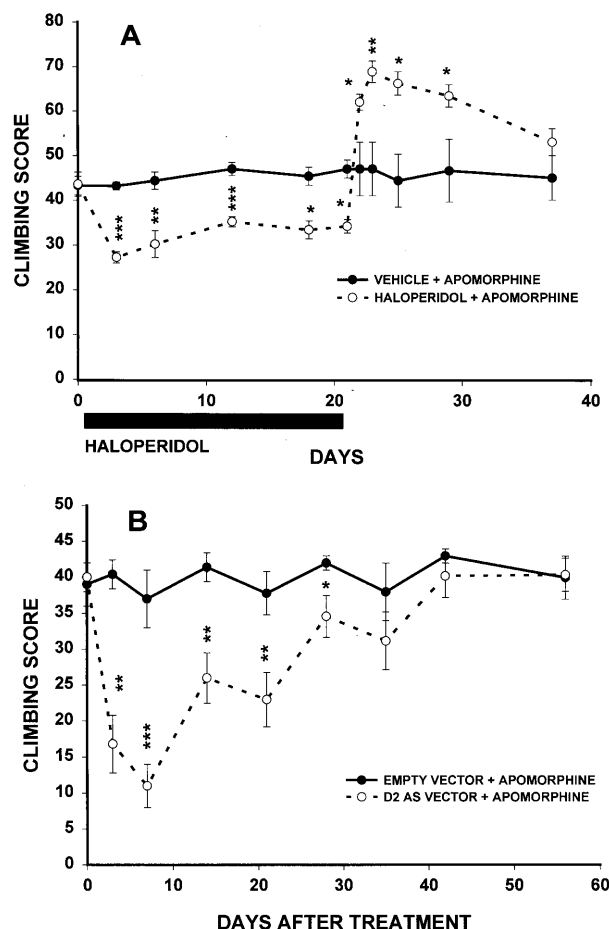

Figure 6. D2 antisense RNA vector, like haloperidol, produces long-term inhibition of apomorphine-induced climbing, but unlike haloperidol, fails to induce a supersensitive dopaminergic response. (*A*) Mice were administered haloperidol (0.6 µmol/kg, intraperitoneally) or vehicle once daily for 21 days. The animals were challenged with apomorphine (16 µmol/kg, subcutaneously) at 3, 6, 12, 18 and 21 days during haloperidol treatment, and at 1, 2, 4, 8 and 16 days after withdrawal from haloperidol treatment, following which apomorphine-induced climbing behavior was measured. (*B*) The D2 antisense RNA vector or empty vector (25 µg each) was injected once, bilaterally into each corpus striatum of mice. At various times thereafter, the animals were challenged with apomorphine (16 µmol/kg, subcutaneously), and climbing behavior was determined. Each point represents the mean value from five mice. Vertical brackets indicate the SE. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with values from mice treated with vehicle or with empty vector. These results show that after withdrawal from chronic treatment with haloperidol there is a supersensitive dopaminergic response (*A*). In contrast, single intrastriatal injections of a D2 antisense RNA expression vector produces long-term inhibition of D2 dopamine receptor-mediated behavior without causing a supersensitive dopaminergic response (*B*). (Taken from [148].)

antagonist haloperidol leads to an upregulation of the D2 dopamine receptors and to an increase or supersensitive response to challenge injections of apomorphine.

In these experiments haloperidol was administered to mice for 21 consecutive days, during which time the animals were given challenge injections with apomorphine, and climbing behavior was determined. The haloperidol treatment was then withdrawn, and the density of dopamine receptors in the corpus striatum and the climbing behavior induced by apomorphine was determined. As may be seen, haloperidol inhibited apomorphine-induced climbing during the period of haloperidol treatment. However, 3 days after withdrawal from haloperidol treatment, the mice evidenced an even greater climbing response to apomorphine than they did before any haloperidol was administered (fig. 6A). This increased dopaminergic behavior was associated with an increased density of D2 dopamine receptors in the corpus striatum [148].

Having established a model of biochemical and behavioral dopaminergic supersensitivity, similar studies were performed using the D2 antisense RNA vector. Single bilateral intrastriatal injections of the D2 antisense vector were administered to mice. The animals were then challenged periodically with apomorphine, and climbing behavior was determined. The results showed that single, bilateral intrastriatal injections of the D2 antisense vector in mice inhibited apomorphine-induced climbing for about 30 days (fig. 6B). The specificity of the observed effects of the D2 antisense vector was ascertained by performing parallel studies with a control empty vector; this vector failed to produce any significant behavioral effects by itself and failed to alter the response to challenge injections with dopamine receptor agonists. The long-term inhibition of the function of dopamine receptors was comparable to that induced by a classical neuroleptic drug, haloperidol (fig. 6A). More important, as the effect of the D2 antisense vector disappeared with time, the climbing response returned to the initial level scored before the injection of the D2 antisense vector. That is, in contrast to that seen after withdrawal from chronic haloperidol treatment (fig. 6A), no increase in apomorphine-induced climbing behavior was ever seen in mice treated with the D2 antisense vector. Moreover, also unlike haloperidol, treatment with the D2 antisense vector did not result in an upregulation in the density of D2 dopamine receptors [148]. These findings suggested that prolonged inhibition of D2 dopamine receptor-mediated behaviors with a D2 antisense vector fails to produce subsequent upregulation of D2 dopamine receptors and does not cause a supersensitive response to the injection of dopamine agonists.

Taken together, these studies suggested that it is possible to produce long-term inhibition of D2 dopamine

receptor functions in vivo by injecting a D2 antisense RNA expression plasmid vector into a discrete brain area. They demonstrated further that this type of treatment may have certain advantages over traditional neuroleptic agents, such as haloperidol, as it would have to be administered less frequently and it would not cause compensatory supersensitive dopaminergic responses.

Effects of antisense RNA vectors targeted to other regulatory proteins. Other studies using vectors expressing antisense RNA in vivo which might be used as potential therapeutic agents for neuropsychiatric disorders include one in which a plasmid vector expressing anti-
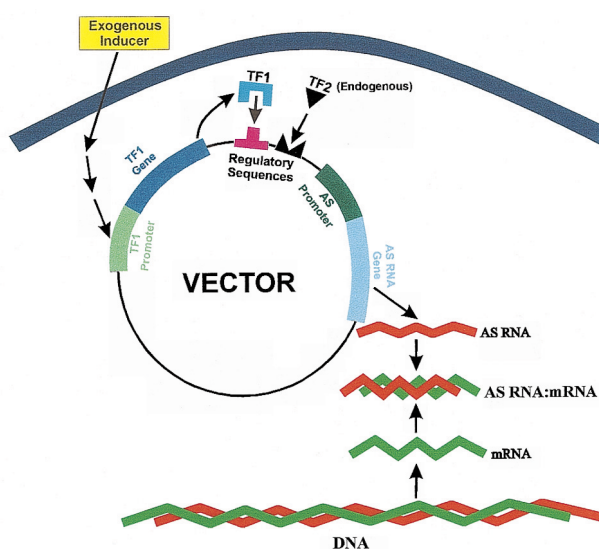

Figure 7. Schematic representation of an antisense RNA-generating vector with tissue-specific and inducible expression of an antisense RNA. The hypothesized antisense RNA-generating vector consists of the following elements: a gene coding for the antisense RNA (AS RNA Gene) and a gene coding for a transcription factor (TF1 Gene), which is necessary for the transcription of the antisense RNA gene. The antisense RNA gene is placed downstream from a promoter (AS Promoter). This promoter, in turn, is activated by two transcription factors (TF1and TF2) that bind to specific DNA sequences (Regulatory Sequences) placed downstream from the AS promoter. One of these transcription factors (TF1) is formed as a result of the activation of an inducible gene (TF1 Gene), which has been inserted along with its promoter (TF1 Promoter) into the same plasmid, and which is induced by an exogenously administered inducer (Exogenous Inducer). The second transcription factor (TF2) is endogenous and must be present only in the targeted cells that contain the targeted mRNA (i.e. it must be cell-specific). Thus, by placing the gene encoding the antisense RNA under the control of a promoter engineered so that it absolutely requires the simultaneous activity of two such different transcription factors, it may be possible to control the expression of the antisense RNA in a specific tissue at any chosen time.

sense RNA to the neuropeptide cholecystokinin octapeptide was injected into the lateral cerebroventricles of rats. The cholecystokinin octapeptide was chosen as a target because it was found that rats that have a relatively small response to the analgesic effects of electroacupuncture have significantly higher levels of cholecystokinin octapeptide than do the high responder rats. The results showed that the administration of this cholecystokinin octapeptide antisense RNA vector was effective in converting the low responder rats into those having a high response to electroacupuncture analgesia and morphine analgesia. These effects began 4 days after the intraventricular injection and lasted for about 1 week. This treatment also delayed the tolerance elicited by prolonged electroacupuncture stimulation or repeated morphine administration [158].

In another study, an antisense to the $GABA_A$ receptor produced by an adeno-associated viral vector was microinjected into the inferior colliculus of rats [159]. This study showed that inhibiting the synthesis of the $GABA_A$ receptor in this brain region using antisense RNA reduced seizure activation in the inferior colliculus. This approach to reduce the expression of $GABA_A$ receptors in certain brain regions and, as a consequence, to reduce their functions, may be applied to the treatment of certain forms of epilepsy.

Outstanding problems and questions. Although progress has been made in the use of antisense RNA in vivo, many problems remain to be solved in order to fully realize the potential of antisense RNA as a clinically useful drug type. Some of these problems are associated with enhancing their uptake and delivery to target tissues, inducing cell-specific and timed expression of the antisense RNA-generating vector and, in cases where the antisense RNA compounds are intended to target transcripts in brain, developing efficient methods to bypass the blood-brain barrier. These problems are likely to be solved using information gleaned not only from studies using vectors expressing antisense RNA but also from gene therapy studies using vectors expressing sense sequences. Other problems are related to understanding in greater detail the mechanisms and the consequences of gene inhibition using antisense RNA. For example, if one targets and blocks the expression of the transcript encoding a given protein, what will be the effect on the transcripts of other proteins? It is also necessary to study in greater detail the pharmacokinetics and possible toxicity of vectors when injected into animals. Finally, the long-term consequences of injecting these DNA-containing antisense RNA expression vectors when used in vivo for therapeutic purposes remain to be determined.

These problems notwithstanding, the major goal is to develop a vector carrying an antisense RNA gene which will not only be selectively expressed in a specific target

tissue but which will be induced to express its antisense RNA for a controlled duration. Such a vector is depicted in figure 7, which describes a hypothetical antisense RNA-generating plasmid vector with tissue-specific and time-controlled expression of antisense RNA. This scheme, which is described in detail in the legend to figure 7, envisions the sequential activation of different promoter elements, and thereby the expression of different genes, all of which are incorporated into the same vector. This process ultimately results in the production of a specific antisense RNA in specific cell types at a specified time. It requires that the targeted cell must contain a tissue-specific transcription factor and that the promoter for the antisense RNA-generating gene must require two different transcription factors: a tissue-specific endogenous transcription factor and one formed as a result of the administration of an exogenous inducer, such as one recently described for tetracycline-responsive promoters [160, 161]. At present there are intense studies searching for suitable promoters for controlled expression, and therefore this model may be modified as new promoters are cloned. An important issue in the choice of such promoters will be the lack of toxicity of the exogenous transcription factor when administered at amounts that induce sufficiently high levels of transcription.

Although numerous questions remain to be solved, achieving the goal of tissue-specific, timed expression of selective antisense RNAs targeted to specific proteins may lead to a new era of molecular pharmaco-therapeutics.

1 Schlingensiepen R., Brysch W. and Schlingensiepen K.-H. (eds) (1997) Antisense from Technology to Therapy, Blackwell Science, Berlin
2 Weiss B. (ed.) (1997) Antisense Oligodeoxynucleotides and Antisense RNA: Novel Pharmacological and Therapeutic Agents, CRC Press, Boca Raton, FL
3 Cohen J. S. (ed.) (1989) Oligodeoxynucleotide Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL
4 Baserga R. and Denhardt D. T. (eds) (1992) Antisense Strategies, New York Academy Sciences, New York
5 Crooke S. T. (ed.) (1995) Therapeutic Applications of Oligonucleotides, R. G. Landes, Austin, TX
6 Raffa R. B. and Porreca F. (eds) (1996) Antisense Strategies for the Study of Receptor Mechanisms, R. G. Landes, Austin, TX
7 Putnam D. A. (1996) Antisense strategies and therapeutic applications. Am. J. Health Syst. Pharm. **53:** 151–160
8 Sczakiel G. (1997) The design of antisense RNA. Antisense Nucl. Acid Drug Dev. **7:** 439–444
9 Oberstrass J. and Nellen W. (1997) Regulating genes with antisense RNA. In: Antisense Oligodeoxynucleotides and Antisense RNA: Novel Pharmacological and Therapeutic Agents, pp. 171–191, Weiss B. (ed.), CRC Press, Boca Raton, FL
10 Mercola D. and Cohen J. S. (1995) Antisense approaches to cancer gene therapy. Cancer Gene Ther. **2:** 47–59
11 Neckers L. and Whitesell L. (1993) Antisense technology: biological utility and practical considerations. Am. J. Physiol. **265:** L1–L12
12 Ohkawa J., Koguma T., Kohda T. and Taira K. (1995) Ribozymes: from mechanistic studies to applications in vivo. J. Biochem. **118:** 251–258
13 Thompson J. D., Macejak D., Couture L. and Stinchomb D. T. (1995) Ribozymes in gene therapy. Nature Med. **1:** 277–278
14 Jaquet V., Gow A., Tosic M., Suchanek G., Breitschopf H., Lassman H. et al. (1996) An antisense transgenic strategy to inhibit the myelin oligodendrocyte glycoprotein synthesis. Molec. Brain Res. **43:** 333–337
15 Crooke S. T. (1997) Advances in understanding the pharmacological properties of antisense oligonucleotides. In: Antisense Oligodeoxynucleotides and Antisense RNA: Novel Pharmacological and Therapeutic Agents, pp. 17–55, Weiss B. (ed.), CRC Press, Boca Raton, FL
16 Wickstrom E. (ed.) (1998) Clinical Trials of Genetic Therapy with Antisense DNA and DNA Vectors, Marcel Dekker, New York
17 Tomita N., Morishita R., Higaki J. and Ogihara T. (1997) A novel strategy for gene therapy and gene regulation analysis using transcription factor decoy oligonucleotides. Exptl. Nephrol. **5:** 429–434
18 Barden N., Stec I. S. M., Montkowski A., Holsboer F. and Reul J. M. H. M. (1997) Endocrine profile and neuroendocrine challenge tests in transgenic mice expressing antisense RNA against the glucocorticoid receptor. Neuroendocrinology **66:** 212–220
19 Gibson S. A. and Shillitoe E. J. (1997) Ribozymes. Their functions and strategies for their use. Mol. Biotech. **7:** 125–137
20 Nielsen P. E. (1998) Antisense properties of peptide nucleic acids. In: Antisense Research and Application, pp. 545–560, Crooke S. T. (ed.), Springer, New York
21 Weiss B., Davidkova G. and Zhang S.-P. (1997) Antisense strategies in neurobiology. Neurochem. Int. **31:** 321–348
22 Qin Z.-H., Zhou L.-W., Zhang S.-P., Wang Y. and Weiss B. (1995) D2 dopamine receptor antisense oligodeoxynucleotide inhibits the synthesis of a functional pool of D2 dopamine receptors. Mol. Pharmacol. **48:** 730–737
23 Weiss B., Zhang S.-P. and Zhou L.-W. (1997) Antisense strategies in dopamine receptor pharmacology. Life Sci. **60:** 433–455
24 Dolnick B. J. (1997) Naturally occurring antisense RNA. Pharmacol. Ther. **75:** 179–184
25 Weiss B., Zhou L.-W. and Zhang S.-P. (1996) Dopamine antisense oligodeoxynucleotides as potential novel tools for studying drug abuse. In: Antisense Strategies for the Study of Receptor Mechanisms, pp. 71–91, Raffa R. and Porreca F. (eds), R. G. Landes, Georgetown, TX
26 Weiss B., Zhou L.-W. and Davidkova G. (1996) Pharmacological and molecular regulation of the expression of dopamine receptors. In: Pharmacological Regulation of Gene Expression in the CNS, pp. 175–196, Merchant K. (ed.), CRC Press, Boca Raton, FL
27 Hou W. F., Zhang S.-P., Davidkova G., Nichols R. A. and Weiss B. (1998) Effect of antisense oligonucleotides directed to individual calmodulin gene transcripts on the proliferation and differentiation of PC12 cells. Antisense Nucl. Acid Drug Dev., in press
28 Zhang S.-P., Nichols R. A. and Weiss B. (1994) Oligodeoxynucleotides antisense to calmodulin mRNAs inhibit proliferation and NGF-induced differentiation of PC12 cells. Soc. Neurosci. **20:** 1316
29 Davidkova G., Zhang S.-P., Zhou L.-W., Nichols R. A. and Weiss B. (1997) Use of antisense RNA expression vectors in neurobiology. In: Antisense Oligodeoxynucleotides and Antisense RNA: Novel Pharmacological and Therapeutic Agents, pp. 213–241, Weiss B. (ed.), CRC Press, Boca Raton, FL

30 Davidkova G., Zhang S.-P., Nichols R. A. and Weiss B. (1996) Reduced level of calmodulin in PC12 cells induced by stable expression of calmodulin antisense RNA inhibits cell growth and induces neurite outgrowth. Neuroscience **75:** 1003–1019

31 Zhang S.-P., Zhou L.-W., Morabito M., Lin R. C. S. and Weiss B. (1996) Uptake and distribution of fluorescein-labeled D2 dopamine antisense oligodeoxynucleotide in mouse brain. J. Mol. Neurosci. **7:** 13–28

32 Davidkova G., Zhang S.-P., Zhou L.-W. and Weiss B. (1998) Effects of dopamine receptor antisense RNA expression vectors in the nervous system. In: Modulating Gene Expression by Antisense Oligonucleotides to Understand Neural Functioning, pp. 61–82, McCarthy M. (ed.), Kluwer Academic Publishers, Norwell, MA

33 Kramm C. M., Sena-Estevez M., Barnett F. H., Rainov N., Schuback D. E., Yu J. S. et al. (1995) Gene therapy for brain tumors. Brain Pathol. **5:** 345–381

34 Smith A. E. (1995) Viral vectors in gene therapy. Ann. Rev. Microbiol. **49:** 807–838

35 Cooper M. J. (1996) Noninfectious gene transfer and expression systems for cancer gene therapy. Semin. Oncol. **23:** 172–187

36 Wilson J.M. (1997) Vectors-shuttle vehicles for gene therapy. Clin. Exptl. Immunol. **107:** 31–32

37 Naldini L., Blomer U., Gallay P., Ory D., Mulligan R. C., Gage F. H. et al. (1996) In vitro gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science **272:** 263–267

38 Yang Y., Li Q., Ertl H. C. and Wilson J. M. (1995) Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. J. Virol. **69:** 2004–2015

39 Byrnes A., MacLaren R. E. and Charlton H. M. (1996) Immunological instability of persistent adenovirus vectors in the brain: peripheral exposure to vector leads to renewed inflammation. J. Neurosci. **16:** 3045–3049

40 Haecker S. E., Stedman H. H., Balice-Gordon R. J., Smith D. B., Greelish J. P., Mitchell M. A. et al. (1996) In vivo expression of full-length human dystrophin from adenoviral vectors deleted of all viral genes. Human Gene Ther. **7:** 1907–1914

41 Gao G. P., Yang Y. and Wilson J. M. (1996) Biology of adenovirus vectors with E1 and E4 deletions for liver-directed gene therapy. J. Virology **70:** 8934–8943

42 Kaplitt M., Leone P., Samulski R., Xiao X., Pfaff D. W., O'Malley K. et al. (1994) Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. Nature Genet. **8:** 148–152

43 Shaughnessy E., Lu D., Saswati C. and Wong K. K. (1996) Parvoviral vectors for gene therapy of cancer. Semin. Oncol. **23:** 159–171

44 Fisher K. J., Kelley W. M., Burda J. F. and Wilson J. M. (1996) A novel adenovirus-adeno-associated virus hybrid vector that displays efficient rescue and delivery of the AAV genome. Human Gene Ther. **7:** 2079–2087

45 Pakzaban P., Geller A. I. and Isacson O. (1994) Effect of exogenous nerve growth factor on neurotoxicity of and neuronal gene delivery by a herpes simplex amplicon vector in the rat brain. Human Gene Ther. **5:** 987–991

46 Sabel B. A., Vick A. and Hoellt V. (1995) Neurotoxic reactions of CNS following gene transfer with defective herpes simplex (HSV-1) vector. Neuroreport **6:** 2447–2450

47 Lasic D. D. and Templeton N. S. (1996) Liposomes in gene therapy. Adv. Drug Deliv. Rev. **20:** 221–266

48 Lappalainen K., Jaaskelainen I., Syrjanen K., Urtti A. and Syrjanen S. (1994) Comparison of cell proliferation and toxicity assays using two cationic liposomes. Pharmaceut. Res. **11:** 1127–1131

49 Parasrampuria D. A. and Hunt C. A. (1998) Therapeutic delivery issues in gene therapy. Part 2. Targeting approaches., Biopharm. May: 58–68

50 Michael S. I. and Curiel D. T. (1994) Strategies to achieve targeted gene delivery via the receptor-mediated endocytosis pathway. Gene Ther. **1:** 223–232

51 Kaneda Y., Iwai K. and Uchida T. (1998) Increased expression of DNA cointroduced with nuclear protein in adult liver. Science **243:** 375–378

52 Gibson I. (1996) Antisense approaches to the gene therapy of cancer – 'Recnac'. Cancer Metast. Rev. **15:** 287–299

53 Hamada K., Sakaue M., Alemany R., Zhang W. W., Horio Y., Roth J. A. et al. (1998) Adenovirus-mediated transfer of HPV 16 E6/E7 antisense RNA to human cervical cancer cells. Gynecol. Oncol. **63:** 219–227

54 Trojan J., Blossey B., Johnson T., Rudin S., Tykocinski M. and Ilan J. (1992) Loss of tumorigenicity of rat glioblastoma directed by episome-based antisense cDNA transcription of insulin-like growth factor I. Proc. Natl. Acad. Sci. USA **89:** 4874–4878

55 Trojan J., Johnson T. R., Rudin S., Ilan J. and Tykocinski M. (1993) Treatment and prevention of rat glioblastoma by immunogenic C6 cells expressing antisense insulin-like growth factor I RNA. Science **259:** 94–97

56 Resnicoff M., Sell C., Rubini M., Coppola D., Ambrose D., Baserga R. et al. (1998) Rat glioblastoma cells expressing an antisense RNA to the insulin-like growth factor-1 (IGF-1) receptor are nontumorigenic and induce regression of wild-type tumors. Cancer Res. **54:** 2218–2222

57 Long L., Rubin R., Baserga R. and Brodt P. (1998) Loss of metastatic phenotype in murine carcinoma cells expressing an antisense RNA to the insulin-like growth factor receptor. Cancer Res. **55:** 1006–1009

58 Trojan J. and Ilan J. (1995) Antisense IGF-1 gene transfer and immunotherapy of glioblastoma. Restor. Neurol. Neurosci. **8:** 77–79

59 Burfeind P., Chernicky C. L., Rininsland F. and Ilan J. (1998) Antisense RNA to the type I insulin-like growth factor receptor suppresses tumor growth and prevents invasion by rat prostate cancer cells in vivo. Proc. Natl. Acad. Sci. USA **93:** 7263–7268

60 Lafarge-Frayssinet C., Duc H. T., Frayssinet C., Sarasin A., Anthony D., Guo Y. et al. (1997) Antisense insulin-like growth factor I transferred into a rat hepatoma cell line inhibits tumorigenesis by modulating major histocompatibility complex I cell surface expression. Cancer Gene Therapy **4:** 276–285

61 Wernert N. (1997) The multiple roles of tumor stroma. Virchows Archiv **430:** 433–443

62 Plate K. and Risau W. (1995) Angiogenesis in malignant gliomas. Glia **15:** 339–347

63 Saleh M., Stacker S. and Wilks A. (1996) Inhibition of growth of C6 glioma cells in vivo by expression of antisense vascular endothelial growth factor sequence. Cancer Res. **56:** 393–401

64 O'Reilly M. S., Boehm T., Shing Y., Fukai N., Vasios G., Lane W. S. et al. (1997) Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell **88:** 277–285

65 Lannutti B. J., Gately S. T., Quevedo M. E., Soff G. A. and Paller A. S. (1997) Human angiostatin inhibits murine hemangioendothelioma tumor growth in vivo. Cancer Res. **57:** 5277–5280

66 Sim B. K., O'Reilly M. S., Liang H., Fortier A. H., He W., Madsen J. W. et al. (1997) A recombinant human angiostatin protein inhibits experimental primary and metastatic cancer. Cancer Res. **57:** 1329–1334

67 Gately S., Twardowski P., Stack M. S., Cundiff D. L., Grella D., Castellino F. J. et al. (1997) The mechanism of cancer-mediated conversion of plasminogen to the angiogenesis inhibitor angiostatin. Proc. Natl. Acad. Sci. USA **94:** 10868–10872

68 Hida K., Shindoh M., Yasuda M., Hanzawa M., Funaoka K., Kohgo T. et al. (1998) Antisense E1AF transfection restrains oral cancer invasion by reducing matrix metalloproteinase activities. Am. J. Pathol. **150:** 2125–2132

69 Yu W., Kim J. and Ossowski L. (1997) Reduction in surface urokinase receptor forces malignant cells into a protracted state of dormancy. J. Cell Biol. **137**: 767–777

70 Ledda M. F., Adris S., Bravo A. I., Kairiyama C., Bover L., Chernajovsky Y. et al. (1997) Suppression of SPARC expression by antisense RNA abrogates the tumorigenicity of human melanoma cells. Nature Med. **3**: 171–176

71 Wu A. G., Joshi S. S., Chan W. C., Iversen P. L., Jackson J. D., Kessinger A. et al. (1995) Effects of BCR-ABL antisense oligonucleotides (AS-ODN) on human myeloid leukemic cells: AS-ODN as effective purging agents. Leukemia Lymphoma **20**: 67–76

72 Rowley P. T., Keng P. C. and Kosciolek B. A. (1996) The effect of bcr-abl antisense oligonucleotides on DNA synthesis and apoptosis in K562 chronic myeloid leukemia cells. Leukemia Res. **20**: 473–480

73 Martiat P., Lewalle A. S., Taj A. S., Philippe M., Larondelle Y., Vaerman J. L. et al. (1993) Retrovirally transduced antisense sequences stably suppress P210 bcr-abl expression and inhibit the proliferation of bcr/abl containing cell lines. Blood **81**: 502–509

74 Jeha S., Luo X. N., Beran M., Kantarjan H. and Atweh G. F. (1998) Antisense RNA inhibition of phosphoprotein p18 expression abrogates the transformed phenotype of leukemic cells. Cancer Res. **56**: 1445–1450

75 Skotzko M., Wu L., French Anderson W., Gordon E. and Hall F. (1995) Retroviral vector-mediated gene transfer of antisense cyclin G1 (CYCG1) inhibits proliferation of human osteogenic sarcoma cells. Cancer Res. **55**: 5493–5498

76 Chen D. S., Zhu N.-L., Hung G., Skotko M. J., Hinton D. R., Tolo V. et al. (1997) Retroviral vector-mediated transfer of an antisense cyclin G1 construct inhibits osteosarcoma tumor growth in nude mice. Hum. Gene Ther. **8**: 1667–1674

77 Hait W. N. and Pierson N. R. (1990) Comparison of the efficacy of a phenothiazine and a bisquinaldinium calmodulin antagonist against multidrug-resistant P388 cell lines. Cancer Res. **50**: 1165–1169

78 Biedler J. L. and Meyers M. B. (1989) Multidrug resistance (Vinca alkaloids, actinomycin D and anthracycline antibiotics). In: Drug Resistance in Mammalian Cells, pp. 125–140, Gupta R. S (ed.), CRC Press, Boca Raton, FL

79 Nagane M., Asai A., Shibui S., Nomura K. and Kuchino Y. (1997) Application of antisense ribonucleic acid complementary to O6-methylguanine-deoxyribonucleic acid methyltransferase messenger ribonucleic acid for therapy of malignant gliomas. Neurosurgery **41**: 434–441

80 Kitajima S., Sano M., Kato K., Seto-Oshima A. and Mizutani A. (1986) Changes of calmodulin and S-100 protein in C6 glioma cells as a result of cellular differentiation induced by forskolin. Acta Histochem. Cytochem. **19**: 365–369

81 MacManus J. P., Gillen M. F., Korczak B. and Nojima H. (1989) Differential calmodulin gene expression in fetal, adult and neoplastic tissues of rodents. Biochem. Biophys. Res. Commun. **159**: 278–282

82 Colomer J., Agell N., Engel P., Alberola-Ila J. and Bachs O. (1993) Calmodulin expression during proliferative activation of human T-lymphocytes. Cell Calcium **14**: 611–621

83 Nojima H. (1989) Structural organization of multiple rat calmodulin genes. J. Mol. Biol. **208**: 269–282

84 Bai G. and Weiss B. (1991) The increase of calmodulin in PC12 cells induced by NGF is caused by differential expression of multiple mRNAs for calmodulin. J. Cell. Physiol. **149**: 414–421

85 Bai G., Nichols R. and Weiss B. (1992) Cyclic AMP selectively up-regulates calmodulin genes I and II in PC12 cells. Biochim. Biophys. Acta **1130**: 189–196

86 Zhang S.-P., Natsukari N., Bai G., Nichols R. and Weiss B. (1993) Localization of the multiple calmodulin messenger RNAs in differentiated PC12 cells. Neuroscience **55**: 571–582

87 Kurpad S., Zhao X.-G., Wikstrand C., Batra S., McLendon R. and Bigner D. (1995) Tumor antigens in astrocytic gliomas. GLIA **15**: 244–256

88 Besnard F., Brenner M., Nakatani Y., Chao R., Purohit H. and Freese E. (1991) Multiple interacting sites regulate astrocyte-specific transcription of the human gene for glial fibrillary acidic protein. J. Biol. Chem. **266**: 18877–18883

89 VandenDriessche T., Chuah M. K. L., Chiang L., Chang H.-K., Ensoli B. and Morgan R. A. (1995) Inhibition of clinical human immunodeficiency virus (HIV) type 1 isolates in primary CD4+ T lymphocytes by retroviral vectors expressing anti-HIV genes. J. Virol. **69**: 4045–4053

90 Bunnell B. A. and Morgan R. A. (1997) Development of retroviral vectors expressing antisense RNA to inhibit replication of the human immunodeficiency virus. In: Antisense Oligodeoxynucleotides and Antisense RNA: Novel Pharmacological and Therapeutic Agents, pp. 198–208, Weiss B. (ed.), CRC Press, Boca Raton, FL

91 VandenDriessche T., Chuah M. K. L. and Morgan R. A. (1994) Gene therapy for acquired immune deficiency syndrome. In: AIDS Updates, pp. 1–14, DeVita V. T., Hellman S. and Rosensberg S. A. (eds), J. B. Lippincott, Philadelphia

92 Peng H., Callison D., Li P. and Burrell C. (1996) Long-term protection against HIV-1 infection conferred by tat or rev antisense RNA was affected by the design of the retroviral vector. Virology **220**: 377–389

93 Junker U., Baker J., Kalfoglou C. S., Veres G., Kaneshima H. and Bohnelein E. (1997) Antiviral potency of drug-gene therapy combinations against human immunodeficiency virus type 1. AIDS Res. Human Retrovir. **13**: 1395–1402

94 Wands J. R., Geissler M., Putlitz J. Z., Blum H., Wezsacker F. V., Mohr L. et al. (1997) Nucleic acid-based antiviral and gene therapy of chronic hepatitis B infection. J. Gastroenterol. Hepatol. **12**: S354–S369

95 Schinke M., Bohm M., Bricca G., Ganten D. and Bader M. (1996) Permanent inhibition of angiotensinogen synthesis by antisense RNA expression. Hypertension **27**: 508–513

96 Phillips M. I. and Gyurko R. (1997) Antisense oligonucleotides targeted to the angiotensin system as a novel strategy to treat hypertension. In: Antisense Oligodeoxynucleotides and Antisense RNA: Novel Pharmacological and Therapeutic Agents, pp. 132–148, Weiss B. (ed.), CRC Press, Boca Raton, FL

97 Phillips M. I., Mohuczy-Dominiak D., Coffey M., Galli S. M., Kimura B., Wu P. et al. (1997) Prolonged reduction of high blood pressure with an in vivo, nonpathogenic, adeno-associated viral vector delivery of AT_1-R mRNA antisense. Hypertension **29**: 374–380

98 Lu D., Yang H. and Raizada M. (1998) Attenuation of ANG II actions by adenovirus delivery of AT1 receptor antisense in neurons and SMC. Am. J. Physiol. **274**: H719–H727

99 Hanna A. K., Fox J. C., Neschis D. G., Safford S. D., Swain J. L. and Golden M. A. (1997) Antisense basic fibroblast growth factor gene transfer reduces neointimal thickening after arterial injury. J. Vascular Surg. **25**: 320–325

100 Harrison P. J. and Burnet P. W. J. (1994) Antisense as an explanatory, experimental and therapeutic tool for psychiatric disorders. Psychol. Med. **24**: 275–279

101 Nicot A. and Pfaff D. W. (1997) Antisense oligonucleotides as specific tools for studying neuroendocrine and behavioral problems: some prospects and problems. J. Neurosci. Meth. **71**: 45–53

102 Zang Z., Florijn W. and Creese I. (1994) Reduction in muscarinic receptors by antisense oligodeoxynucleotide. Biochem. Pharmacol. **48**: 225–228

103 Zhang S.-P., Zhou L.-W. and Weiss B. (1994) Oligodeoxynucleotide antisense to the D1 dopamine receptor mRNA inhibits D1 dopamine receptor-mediated behaviors in normal mice and in mice lesioned with 6-hydroxydopamine. J. Pharmacol. Exp. Ther. **271**: 1462–1470

104 Weiss B., Zhou L.-W., Zhang S.-P. and Qin Z.-H. (1993) Antisense oligodeoxynucleotide inhibits D2 dopamine receptor-mediated behavior and D_2 messenger RNA. Neuroscience **55**: 607–612

105 Zhou L.-W., Zhang S.-P., Qin Z.-H. and Weiss B. (1994) In vivo administration of an oligodeoxynucleotide antisense to the D₂ dopamine receptor mRNA inhibits D₂ dopamine receptor-mediated behavior and the expression of D₂ dopamine receptors in mouse striatum. J. Pharmacol. Exp. Ther. **268:** 1015–1023

106 Zhou L.-W., Zhang S.-P. and Weiss B. (1996) Intrastriatal administration of an oligodeoxynucleotide antisense to the D2 dopamine receptor mRNA inhibits D2 dopamine receptor-mediated behavior and D2 dopamine receptors in normal mice and in mice lesioned with 6-hydroxydopamine. Neurochem. Int. **29:** 583–595

107 Zhang M. and Creese I. (1993) Antisense oligodeoxynucleotide reduces brain dopamine D₂ receptors: behavioral correlates. Neurosci. Lett. **161:** 223–226

108 Silvia C. P., King G. R., Lee T. H., Xue Z.-Y., Caron M. G. and Ellinwood E. H. (1994) Intranigral administration of D₂ dopamine receptor antisense oligodeoxynucleotides establishes a role for nigrostriatal D₂ autoreceptors in the motor actions of cocaine. Mol. Pharmacol. **46:** 51–57

109 Tremblay M., Rouillard C. and Levesque D. (1997) Dopamine D3 receptor antisense reduces neuropeptide mRNA levels in rat nucleus accumbens. Neuroreport **8:** 3901–3905

110 Nissbrandt H., Ekman A., Eriksson E. and Heilig M. (1995) Dopamine D3 receptor antisense influences dopamine synthesis in rat brain. Neuroreport **6:** 573–576

111 Dziewczapolski G., Menalled L., Garcia M., Mora M., Gershanik O. and Rubenstein M. (1998) Opposite roles of D1 and D5 dopamine receptors in locomotion revealed by selective antisense oligonucleotides. Neuroreport **9:** 1–5

112 Wahlestedt C., Golanov E., Yamamoto S., Yee F., Ericson H., Yoo H. et al. (1993) Antisense oligodeoxynucleotides to NMDA-R1 receptor channel protect cortical neurons from excitotoxicity and reduce focal ischaemic infarctions. Nature **363:** 260–263

113 Sun F. Y. and Faden A. I. (1995) Pretreatment with antisense oligodeoxynucleotides directed against the NMDA-R1 receptor enhances survival and behavioral recovery following traumatic brain injury in rats. Brain Res. **693:** 163–168

114 Sleight A. J., Monsma F. J. Jr., Borroni E., Austin R. H. and Bourson A. (1995) Effects of altered 5-ht₆ expression in the rat: functional studies using antisense oligonucleotides., Behav. Brain Res. **73:** 245–248

115 Gyurko R., Wielbo D. and Phillips M. I. (1993) Antisense inhibition of AT₁ receptor mRNA and angiotensinogen mRNA in the brain of spontaneously hypertensive rats reduces hypertension of neurogenic origin. Regul. Pept. **49:** 167–174

116 Sakai R. R., He P. F., Yang X. D., Ma L. Y., Guo Y. F., Reilly J. J. et al. (1994) Intracerebroventricular administration of AT1 receptor antisense oligonucleotides inhibits the behavioral actions of angiotensin II. J. Neurochem. **62:** 2053–2056

117 Meng H., Wielbo D., Gyurko R. and Phillips M. I. (1994) Antisense oligonucleotide to AT₁ receptor mRNA inhibits central angiotensin induced thirst and vasopressin. Regul. Pept. **54:** 543–551

118 Ambühl P., Gyurko R. and Phillips M. I. (1995) A decrease in angiotensin receptor binding in rat brain nuclei by antisense oligonucleotides to the angiotensin AT₁ receptor. Regul. Pept. **59:** 171–182

119 Vanderah T. W., Lai J., Yamamura H. and Porreca F. (1994) Antisense oligodeoxynucleotide to the CCKb receptor produces naltrindole- and [Leu5] enkephalin antiserum-sensitive enhancement of morphine antinociception. Neuroreport **5:** 2601–2605

120 Wahlestedt C., Pich E. M., Koob G. F., Yee F. and Heilig M. (1993) Modulation of anxiety and neuropeptide Y-Y1 receptors by antisense oligodeoxynucleotides. Science **259:** 528–531

121 Ogo H., Hirai Y., Miki S., Nishio H., Akiyama M. and Nakata Y. (1994) Modulation of substance P/neurokinin-1 receptor in human astrocytoma cells by antisense oligodeoxynucleotides. Gen. Pharmacol. **25:** 1131–1135

122 Skutella T., Probst J. C., Engelmann M., Wotjak C. T., Landgraf R. and Jirikowski G. F. (1994) Vasopressin antisense oligonucleotide induces temporary diabetes insipidus in rats. J. Neuroendocrinol. **6:** 121–125

123 Standifer K. M., Chien C.-C., Wahlestedt C., Brown G. P. and Pasternak G. W. (1994) Selective loss of δ opioid analgesia and binding by antisense oligodeoxynucleotides to a δ opioid receptor. Neuron **12:** 805–810

124 Adams J. U., Chen X., deRiel J. K., Adler M. W. and Liu-Chen L.-Y. (1994) Intracerebroventricular treatment with an antisense oligodeoxynucleotide to kappa-opioid receptors inhibited kappa-agonist-induced analgesia in rats. Brain Res. **667:** 129–132

125 Rossi G., Pan Y.-X., Cheng J. and Pasternak G. W. (1994) Blockade of morphine analgesia by an antisense oligodeoxynucleotide against the mu receptor. Life Sci. **54:** PL375–PL379

126 Lai J., Bilsky E. J. and Porreca F. (1995) Treatment with antisense oligodeoxynucleotide to a conserved sequence of opioid receptors inhibits antinociceptive effects of delta subtype selective ligands. J. Recept. Res. **15:** 643–650

127 McCarthy M. M., Schlenker E. H. and Pfaff D. W. (1993) Enduring consequences of neonatal treatment with antisense oligonucleotides to estrogen receptor messenger ribonucleic acid on sexual differentiation of rat brain. Endocrinology **133:** 433–439

128 Pollio G., Xue P., Zanisi M., Nicolin A. and Maggi A. (1993) Antisense oligonucleotide blocks progesterone-induced lordosis behavior in ovariectomized rats. Mol. Brain Res. **19:** 135–139

129 Ogawa S., Olazábal U. E., Parhar I. S. and Pfaff D. W. (1994) Effects of intrahypothalamic administration of antisense DNA for progesterone receptor mRNA on reproductive behavior and progesterone receptor immunoreactivity in female rat. J. Neurosci. **14:** 1766–1774

130 Sokoloff P. and Schwartz J.-C. (1995) Novel dopamine receptors half a decade later. Trends Pharmacol. Sci. **16:** 270–275

131 Gingrich J. and Caron M. (1993) Recent advances in the molecular biology of dopamine receptors. Ann. Rev. Neurosci. **16:** 299–321

132 Civelli O., Bunzow J. R., Grandy D. K., Zhou Q.-Y. and Van Tol H. H. M. (1991) Molecular biology of the dopamine receptors. Eur. J. Pharmacol. **207:** 277–286

133 Seeman P. (1987) Dopamine receptors and the dopamine hypothesis of schizophrenia. Synapse **1:** 133–152

134 Lewis D. A., Hayes T. L., Lund J. S. and Oeth K. M. (1992) Dopamine and the neural circuitry of primate prefrontal cortex: implications for schizophrenia research. Neuropsychopharmacology **6:** 127–1341

135 Carlsson A. and Piercey M. (eds) (1995) Dopamine Receptor Subtypes in Neurological and Psychiatric Diseases, Raven Press, New York

136 Kuhar M. J., Ritz M. C. and Boja J. W. (1991) The dopamine hypothesis of the reinforcement properties of cocaine. Trends Neurosci. **14:** 299–302

137 Fibiger H. C., Phillips A. G. and Brown E. E. (1992) The neurobiology of cocaine-induced reinforcement. Ciba Found. Symp. **166:** 96–111

138 Gorwood P., Ades J. and Feingold J. (1994) Are genes coding for dopamine receptors implicated in alcoholism? Eur. Psychiatry **9:** 63–69

139 Seeman P., Bzowej N. H., Guan H.-C., Bergeron C., Reynolds G. P., Bird E. D. et al. (1987) Human brain D1 and D2 dopamine receptors in schizophrenia, Alzheimer's, Parkinson's and Huntington's diseases. Neuropsychopharmacology **1:** 5–15

140 Korf J. (1997) Imaging of receptors in clinical neurosciences. Acta Neurol. Belgica **97:** 200–205

141 Weeks R. A., Piccini P., Harding A. E. and Brooks D. J. (1996) Striatal D1 and D2 dopamine receptor loss in asymptomatic mutation carriers of Huntington's disease. Ann. Neurol. **40:** 49–54

142 Casey D. E. (1991) Neuroleptic drug-induced extrapyramidal syndromes and tardive dyskinesia. Schizophr. Res. **4:** 109–120

143 Lublin H., Gerlach J. and Peacock L. (1992) Effect of D_1 and D_2 agonists in primates withdrawn from long-term treatment with haloperidol: the potential role of dopamine D_1 receptors in dyskinesia. Clin. Neuropharmacol. **15:** 448–458

144 Marken P. A., Haykal R. F. and Fisher J. N. (1992) Management of psychotropic-induced hyperprolactinemia. Clin. Pharmacy **11:** 851–856

145 Braghiroli L., Ponzianelli A., Fiani M., De Feo G., Cheng C. Y. and Mazzanti G. (1997) Effect of clozapine on dopamine-induced inhibition of prolactin release from cultured rat pituitary cells. Pharmacol. Res. **35:** 173–175

146 Weiss B., Davidkova G., Zhou L.-W., Zhang S.-P. and Morabito M. (1997) Expression of D2 dopamine receptor antisense RNA in brain inhibits D2-mediated behaviors. Neurochem. Int. **31:** 571–580

147 Boundy V. A., Luedtke R. R. and Molinoff P. B. (1993) Development of polyclonal anti-D2 dopamine receptor antibodies to fusion proteins: inhibition of D2 receptor-G protein interaction. J. Neurochem. **60:** 2181–2191

148 Davidkova G., Zhou L.-W., Morabito M., Zhang S.-P. and Weiss B. (1998) D2 dopamine antisense RNA expression vector, unlike haloperidol, produces long-term inhibition of D2 dopamine-mediated behaviors without causing up-regulation of D2 dopamine receptors. J. Pharmacol. Exptl. Ther. **285:** 1187–1196

149 Savasta M., Dubois A., Feuerstein C., Manier M. and Scatton B. (1987) Denervation supersensitivity of striatal D2 dopamine receptors is restricted to the ventro- and dorsolateral regions of the striatum. Neurosci. Lett. **74:** 180–186

150 Winkler J. D. and Weiss B. (1986) Reversal of supersensitive apomorphine-induced rotational behavior in mice by continuous exposure to apomorphine. J. Pharmacol. Exp. Ther. **238:** 242–247

151 Weiss B., Zhou L.-W., Chen J. F., Szele F. and Bai G. (1990) Distribution and modulation of the D2 dopamine receptor mRNA in mouse brain: molecular and behavioral correlates. Adv. Biosci. **77:** 9–25

152 Weiss B. and Costa E. (1967) Adenyl cyclase activity in rat pineal gland: effects of chronic denervation and nore-pinephrine. Science **156:** 1750–1752

153 Weiss B. and Kidman A. (1969) Neurobiological significance of cyclic 3′,5′-adenosine monophosphate. In: Advances in Biochemical Pharmacology, pp. 131–164, Costa E. and Greengard P. (eds), Raven Press, NY

154 Weiss B. and Strada S. (1972) Neuroendocrine control of the cyclic AMP system of brain and pineal gland. In: Advances in Cyclic Nucleotide Research, vol. 1, pp. 357–374, Greengard P., Robinson G. A. and Paoletti R. (eds), Raven Press, NY

155 Creese I. and Snyder S. H. (1998) Receptor adaptations to centrally acting drugs. Ann. Rev. Pharmacol. **21:** 357–391

156 Rogue P., Hanauer A., Zwiller J., Malviya A. N. and Vincendon G. (1991) Up-regulation of dopamine D2 receptor mRNA in rat striatum by chronic neuroleptic treatment. Eur. J. Pharmacol. **207:** 165–168

157 Weiss B. and Davidkova G. (1996) Cyclic nucleotides. In: Encyclopedia of Neuroscience, pp. 1–4, Adelman G. and Smith B. (eds), Elsevier Science, Amsterdam

158 Tang N. M., Dong H. W., Wang X. M., Tsui Z. C. and Han J. S. (1997) Cholecystokinin antisense RNA increases the analgesic effect induced by electroacupuncture or low dose morphine: conversion of low responder rats into high responders. Pain **71:** 71–80

159 Xiao X., McCown T., Li J., Breese G. R., Morrow A. L. and Samulski R. J. (1997) Adeno-associated virus (AAV) vector antisense gene transfer in vivo decreases GABA(A) alpha 1-containing receptors and increases inferior collicular seizure sensitivity. Brain Res. **756:** 76–83

160 Harding T. C., Geddes B. J., Murphy D., Knight D. and Uney J. B. (1998) Switching transgene expression in the brain using an adenoviral tetracycline-regulatable system. Nature Biotech. **16:** 553–555

161 Sapolsky R. M., Ho D. Y. and McLaughlin J. (1998) The ins and outs of on and off. Nature Biotech. **16:** 516–517

162 Singh P., Owlia A., Varro A., Dai B., Rajaraman S. and Wood T. (1998) Gastrin gene expression is required for the proliferation and tumorigenicity of human colon cancer cells. Cancer Res. **56:** 4111–4115

163 Li Y. and Wang Y. (1997) Inhibition growth of multidrug resistant KBV200 cells by MDR1 antisense RNA. Biochem. Biophys. Res. Commun. **239:** 345–348

164 Ahmad S., Mineta T., Martuza R. and Glazer R. (1994) Antisense expression of protein kinase C alpha inhibits the growth and tumorigenicity of human glioblastoma cells. Neurosurgery **35:** 904–909