A RECENT report from the Recombinant DNA Advisory Committee lists 393 approved human gene therapy protocols (www.od.nih.gov/oba/documents.htm). Of these, 33 are for infectious diseases, 49 for monogenic diseases (mostly cystic fibrosis), 237 for cancer, 35 for other disorders, and the remainder for nontherapeutic trials. Despite the serious nature of pain, both in terms of human suffering and economic cost, there are no human gene therapy protocols that target it. Could it be that the biologic basis of pain is too ill defined to apply the state-of-art gene therapy to it? In recent years, significant progress has been made in our fundamental understanding of the neurobiology of pain. In parallel, detailed molecular biologic characterization of many of the receptors and ion channels playing significant roles in nociception have become available. Although in its infancy, gene therapy, or the translational clinical application of the molecular biologic revolution, has fundamentally changed the way we study basic biologic processes and clearly will impact our clinical management of diverse diseases, including pain.

This review summarizes recent developments in gene therapy and demonstrates the possibility of gene therapy for the management of pain. This is a broad topic requiring expertise in diverse areas of science and medicine. As such, diverse literature spanning basic molecular biology, viral vectors, antisense oligonucleotide, and pain literature was reviewed. We have strived to create a review with a dual mission of serving as a didactic document and as a document that will serve as a reference for active investigators in this area of science. Most importantly, we hope that this review will stimulate the interest of the readers to further explore the genetic approach to pain management. In Part I, we begin with a discussion of strategies for gene therapy, followed by an introduction of the fundamental methods and technologies (both viral and nonviral) available for gene therapy and transfer. Part II of the review, to be published subsequently, will describe potential nociceptive targets for the methods and strategies of gene therapy described in this review.

The pathways of nociception are not summarized; however, there are many excellent reviews summarizing recent developments in our understanding of pain mechanisms. An authoritative review covering neuronal pathways to molecules mediating pain was recently published.7 Our goal is to minimize overlap with these reviews by focusing on information pertinent for gene therapy considerations. Readers unfamiliar with the basic molecular biologic concepts are referred to an excellent primer on molecular biology recently published in ANESTHESIOLOGY or chapters in standard reference books.9-11 Specific protocols for routine molecular biologic techniques can be found in any of the laboratory manuals,12,13 and practical methods for creating gentransduction viral vectors can be found in references cited in table 1. Reviews by Eck and Wilson,14 Andersen,15 and Kay et al.16 provide excellent general overviews of gene therapy.

Lastly, clinical trials using gene therapeutic agents are subject to full federal regulations, as with any new investigational drug. Moreover, gene therapy protocols are subject to an additional oversight by the federal recombinant DNA advisory committee. Further information and guidelines for developing a human clinical trial protocol, as well as an updated list of active gene therapy trials, can be found at the National Institutes of Health web site referenced above.
gene regulation in eukaryotes, many of which are potentially target antinociceptive targets such as the N-methyl-D-aspartate receptor, protein kinase C, and neurokinin 1 receptors. Successful expression of functional protein product requires transfer and expression of a gene encoding the entire functional target protein, which is best accomplished by viral vector technology. Alternative technologies capable of transfer of a large DNA include the gene gun for direct injection of plasmid constructs into target tissues and a lipid-carrier-mediated transfection of plasmid constructs and a viral protein-plasmid complex uptake. A recent review discusses these and other synthetic DNA delivery systems.

A second strategy already in active clinical trials is an antisense oligonucleotide-mediated knockdown of target protein levels presumably through enhanced mRNA degradation (i.e., a gene-product deletion therapy). This knockdown strategy targets disease-causing or potential pronociceptive targets, such as N-methyl-D-aspartate receptor, protein kinase C, and neurokinin 1 receptors. Administration of a short 18–20-mer oligonucleotide with a sequence complimentary to the gene encoding the target protein results in a decrease in protein levels. Although the precise mechanisms responsible for antisense knockdown remain unknown, translational arrest caused by activity of a specific cellular enzyme (RNAse H)-mediated degradation of double-stranded hybrid mRNA and transcriptional arrest through interference with unwinding of double-stranded gene have been proposed. This approach is limited in that antisense oligonucleotide-mediated therapy attempts protein down-regulation. Therefore, only pronociceptive targets are amenable to therapeutic intervention. In addition to the antisense oligonucleotide approach, knockdown of protein target can be accomplished using a viral vector designed to transcribe mRNA in the antisense orientation as well.

A parallel approach for expression of diffusible antinociceptive molecules through transplantation of ex vivo transduced cells back into the intact organism is an attractive approach to gene product therapy but is not

### Strategies for Gene Therapy

In this review, gene therapy is used synonymously with somatic cell gene therapy as opposed to germ line gene therapy. The distinction lies in the fact that somatic cell gene therapy targets somatic cells. The gene transfer and its therapeutic (or deleterious) effects end when the organism dies. All clinical gene therapy protocols approved to date involve somatic cell gene therapy. In germ line gene therapy, genetic manipulation passes on to the progeny. Creation of animals devoid of a certain gene (i.e., gene knockout animals) is being increasingly used in experimental work to investigate the functional significance of a given gene product. The power of such a genetic approach to decipher mechanisms underlying pathologic pain for experimental purposes is clear; however, application of germ line gene therapy for creating organisms that will never develop pathologic pain is neither therapeutically desirable nor ethically acceptable. Gene therapy attempts to regulate expression of a target protein based on our understanding of how cells regulate gene expression. There are six general levels of gene regulation in eukaryotes, many of which are potentially amenable to artificial control and thus potentially useful for gene therapy. These levels include transcriptional control, RNA processing control, RNA transport control, mRNA degradation control, translational control, and protein activity control. In theory, any of these points of gene regulation, alone or in combination, can be targeted by gene therapy. Thus far, practical application of gene therapy has been limited mainly to an expression of a lacking or defective protein by a self-contained gene-expression cassette (i.e., a gene-product replacement therapy). This approach is most often seen in gene therapy for a monogenic disease (e.g., adenosine deaminase deficiency and cystic fibrosis). The overexpression strategy for pain management could potentially target antinociceptive targets such as the acetylcholine, cannabinoid, opioid, and serotonin receptors. Successful expression of functional protein product requires transfer and expression of a gene encoding the entire functional target protein, which is best accomplished by viral vector technology. Alternative technologies capable of transfer of a large DNA include the gene gun for direct injection of plasmid constructs into target tissues and a lipid-carrier-mediated transfection of plasmid constructs and a viral protein-plasmid complex uptake. A recent review discusses these and other synthetic DNA delivery systems.

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A parallel approach for expression of diffusible antinociceptive molecules through transplantation of ex vivo transduced cells back into the intact organism is an attractive approach to gene product therapy but is not
Table 2. Viral Vectors for Central Nervous System Gene Therapy*

<table>
<thead>
<tr>
<th>Vector</th>
<th>Genome Size (kb)</th>
<th>Insert Capacity (kb)</th>
<th>Episomal or Integrating</th>
<th>Immunogenicity</th>
<th>Diameter of Viral Particle (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA virus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herpes simplex</td>
<td>152</td>
<td>15</td>
<td>Episomal</td>
<td>High</td>
<td>200</td>
</tr>
<tr>
<td>HSV ampiclon</td>
<td>†</td>
<td>†</td>
<td>Episomal</td>
<td>Low</td>
<td>80</td>
</tr>
<tr>
<td>Adenovirus—E1-deleted‡</td>
<td>36</td>
<td>7</td>
<td>Episomal</td>
<td>High</td>
<td>20</td>
</tr>
<tr>
<td>Adenovirus—gutless§</td>
<td>§</td>
<td>§</td>
<td>Episomal</td>
<td>Low</td>
<td>80</td>
</tr>
<tr>
<td>Adeno-associated</td>
<td>4.7</td>
<td>4.8</td>
<td>Integrating</td>
<td>Low</td>
<td>20</td>
</tr>
<tr>
<td>Retrovirus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maloney leukemia#</td>
<td>9</td>
<td>5</td>
<td>Integrating</td>
<td>Low</td>
<td>100</td>
</tr>
<tr>
<td>HIV</td>
<td>7</td>
<td>7</td>
<td>Integrating</td>
<td>Low</td>
<td>80</td>
</tr>
<tr>
<td>RNA virus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sindbis**</td>
<td>11.7</td>
<td>3</td>
<td>Exists as RNA</td>
<td>High</td>
<td>40</td>
</tr>
</tbody>
</table>

* Viral vectors are potentially hazardous and necessitate institutional biosafety approval before work is commenced. Replication-incompetent vectors can be created safely at the biosafety level II precautions no different from routine work on cells of human origin. General information on the biosafety level recommended for recombinant DNA work can be found at http://www4.od.nih.gov/oba/ (National Institutes of Health, accessed April 12, 2001).
† DNA inserted in the herpes simplex virus (HSV) ampiclon is replicated as a head-to-tail concatemer of approximately 150 kb, which approximates the size of the wild-type HSV genome. In theory, a transgene as large as the full 150 kb should be transducible. In practice, limitation of stability of plasmid-based ampiclon limits the payload to 15 kb. Use of large-capacity cloning vehicles, such as cosmids or yeast artificial chromosomes, should improve the payload capacity dramatically.
‡ E1-, E3-deleted adenoviral vectors (shuttle and parent plasmids) are available from Microbix Inc. (http://www.microbix.com, accessed April 12, 2001) and Quantum Biotechnologies Inc. (http://www.quantumbiotech.com, accessed April 12, 2001). Creation of a recombinant adenovirus using the commercial reagents requires subcloning followed by homologous recombination in HEK293 cells. Further cesium chloride gradient centrifugation followed by dialysis is required to obtain clean adenovirus. Although marketed as a “kit,” creation of an adenoviral vector—harboring gene of interest is technically demanding, requiring considerable expertise in molecular biology and cell culture. Custom services for creating a specific recombinant E1-, E3-deleted adenovirus is commercially available but quite costly. An excellent laboratory manual for creating first-generation adenovirus can be found in Ehrenbruber et al.124 or in Graham and Prevec.125 Further technical reference for creating recombinant adenovirus can be found on the Microbix Inc. Web site.
§ For gutless adenovirus, total DNA (i.e., genome size) must be between 28 and 36 kb for efficient packaging into viral particles. For small transgenes, including the promoter and adenylation signals, the remainder of the space is filled with unrelated DNA (often λ-phage DNA) to meet the minimum size requirement. In theory, multiple-expression cassettes that use individual promoters or are concatenated by the internal ribosomal entry site sequence can be used instead of the phage DNA to create a vector expressing multiple therapeutic genes simultaneously. Therefore, the theoretical insert capacity can approach 36 kb.
|| Reagents for aden-associated adenovirus vector creation are not available as a kit. However, plasmids harboring the aden-associated virus (catalog No. 37216, 68065) necessary for creating recombinant aden-associated virus construct are available from American Type Culture Collection Inc. (http://atcc.org, accessed April 12, 2001). A good protocol can be found in Skulimowski and Simulski.126
¢ A series of murine Moloney leukemia virus—derived vectors and packaging cell lines are available as a kit from Clontech Inc. (http://atcc.org, accessed April 12, 2001). Creation of a murine Moloney leukemia virus—based retrovirus requires subcloning of the gene of interest into the commercially available vector and transient transfection into a packaging cell line. Infective but replication-incompetent retrovirus can be harvested from the cell culture supernatant. The protocol is well-documented and technically easy. We were unable to locate a specific reference stating the upper limit of insert size. However, because murine Moloney leukemia virus vectors essentially lack the entire viral genome, insert approaching the wild-type viral genome size should be packagable. The 5-kb payload limit listed in the table was obtained from Clontech Technical Support.
** A Sindbis virus—derived vector and a helper virus DNA can be purchased from Invitrogen Inc. (http://www.invitrogen.com, accessed April 12, 2001). Creation of a Sindbis virus requires subcloning of the gene of interest into the pShIV vectors and in vitro transcription of RNA from both the pShIV and helper vectors, followed by transfection into a baby hamster kidney—permissive cell line. In vitro transcription is tricky for those not used to working with RNA. Otherwise, the procedure as described in the package insert is straightforward. We noted that dialysis of cell culture supernatant before usage reduces direct cytotoxicity of Sindbis virus. The virus can package exogenous inserts of 3 kb; however, the payload is limited by insert stability, with inserts of less than 2 kb exhibiting greatest stability (see Huang127). An indispensable source of information for α-virus vectors can be found at http://www.microbiology.wustl.edu/sindbis/αVectors (Washington University School of Medicine, Department of Microbiology, accessed April 12, 2001).

discussed in this review. In theory, patient’s own cells could be collected, engineered to produce antinociceptive molecules, and subsequently transplanted back into the central nervous system (CNS), eliminating immunologic problems associated with xenografts. Further details of this approach can be found in an excellent review by Czech and Sagen.20

Tools for Gene Therapy

Gene therapy, in contrast to conventional pharmacologic therapy, targets DNA or RNA to attain its therapeutic goal. Whether it involves transfer of a short segment of DNA (antisense oligonucleotide method) or full-length cDNA encoding the entire functional protein, the initial step of gene therapy depends on successful transfer of the “gene-targeting drug.” In this section, two specific somatic cell gene transfer techniques, the viral vector and antisense oligonucleotide methods, are discussed, as these approaches represent, in our view, technologies closest to human clinical applications. Advantages and disadvantages of the two principal methods are summarized in table 1. Space limitation precludes discussion of other approaches to gene (product) transfer, including ribozymes,21 gene gun,17 and transplantation of gene—targeted cells.20 We focus our discussion on methods effecting CNS targets. The same methodology could be applied to novel peripheral targets involved in pain, such
as the axons and cell bodies of the autonomic and peripheral nervous systems, and non-nervous system targets such as the epithelial cells responsible for the mechano-transduction at free nerve terminals. Our intent is not to minimize the potential utility of peripheral targets, but to focus on the CNS targets, particularly in the spinal cord, because such targets should be amenable to focal therapy through direct intrathecal intervention. Focal therapy is highly desirable because the risk of undesired side effects may have a serious consequence when manipulating genetic targets.

Viral Vectors

Development of Recombinant Vectors. Traditional methods of gene transfer, such as calcium phosphate precipitation, electroporation, or various lipid carrier-mediated techniques, do not work with high efficiency in postmitotic cells and are mostly impractical for CNS applications. Recently developed viral vectors provide a powerful vehicle for effective CNS gene transfer. Through evolution, viruses have acquired the ability to infect and transduce their genome into host cells. The term transduction refers to the process by which a virus infects a host cell and introduces its genetic contents. Viral vector–based gene therapy takes advantage of the natural biology of virus to transduce exogenous genes into host cells. The general principle for creating a viral vector is to delete nonessential genes from the virus, incapacitating its replication and therefore rendering it nonpathogenic yet retaining the ability to attach and transduce its genome into the host. However, rendering a virus replication deficient does not assure elimination of direct cytotoxicity or abolition of host immune response caused by expression of other viral proteins not critical for replication. An ideal vector for gene therapy should achieve stable, tissue-specific regulated gene expression without eliciting the host immune response. The choice of which viral vector to be used is based also on the following practical transgene-specific considerations: (1) What is the size of the exogenous gene one wants to transfer? (2) Is the target cell mitotically active (e.g., glial cells) or postmitotic (e.g., nerve cells)? (3) What is the desired route of delivery? and (4) What is the desired duration of therapeutic intervention?

The size of gene to be transferred determines the required “payload” capacity of the viral vector. In general, a virus with a larger native genome is capable of carrying a larger exogenous gene. A DNA replication mechanism, such as the rolling replication system used by the herpes simplex virus (HSV) amplified concatemers (amplicon), allows for inherent amplification of the exogenous gene, in effect increasing the gene dose. The desired target of gene transfer defines whether the viral vector must transduce nondividing cells. For CNS gene therapy, where the desired target is most often postmitotic nerve cells, use of vectors such as the Ma-loney murine leukemia virus (MMLV) and adeno-associated virus (AAV), which have limited ability to infect mitotically silent cells, is precluded. Lastly, the desired duration of the exogeneous gene expression specifies whether an integrating or nonintegrating viral vector is needed. Transduction with integrating virus (e.g., human immunodeficiency virus [HIV]) results in integration of the exogenous gene into the host genome, thereby assuring perpetual presence of the exogenous gene in future generations of cells resulting from mitosis. Non-integrating virus (e.g., adenovirus and HSV) allows only an extrachromosomal existence of the exogenous gene, resulting in the eventual loss of the transgene with cell division even during ideal circumstances.

DNA virus, RNA virus, and retrovirus have all been used as experimental gene-transduction vectors (table 2). The four most commonly used viral vectors are based on MMLV, AAV, adenovirus, and HSV. The MMLV, a retrovirus, was the first to enter a human clinical trial; however, because of its poor transduction efficiency and inability to infect postmitotic cells, the utility of MMLV for CNS gene therapy is limited with the exception of its use in transducing mitotically active cells ex vivo for subsequent transplantation into CNS. Retrovirus introduced into an organism as a gene therapy vector also carries the theoretical risk of insertional mutagenesis. Recombination with stably integrated retroviral sequences present in the human genome, resulting in the creation of a replication-competent retrovirus, is also possible. Despite these theoretical limitations, MMLV-based gene therapy has demonstrated clinical efficacy for patients with a monogenic disease. Advent of an HIV-based vector with the ability to transduce neurons may pave the way for further use of retrovirus (lenti-virus) in CNS gene therapy as well.

Recent progress in AAV production methods allows creation of high-titer vectors with essentially no helper-adenovirus contamination. Several laboratories have now confirmed successful persistent AAV-mediated transgene expression in brain and spinal cord without cytopathic effects, refuting the traditional notion that AAVs are incapable of transducing postmitotic cells; however, the limited insert capacity of this virus restricts its use to transfer of small transgenes. A hybrid adenovirus–MMLV or an adenovirus–AAV construct that cloaks the retrovirus or AAV sequence within the adenovirus, resulting in a stably integrating virus with a broad host range, may be a particularly attractive vector for long-term genetic intervention required for management of chronic pain.

The HSV was the first to offer a large cloning capacity enabling expression of large transgenes. Its natural neurotropic property makes this vector especially attractive for CNS gene delivery. The major disadvantage is the lack of understanding of the complex viral biology regulating entry into the latent phase where viral gene
expression (and presumably the transgene which one wants to express) ceases. As with the AAV, the present requirement for a helper virus for propagation and large-scale preparation of recombinant HSV imposes some limitations as well. A recent cosmid library-based helper-virus-free packaging of defective HSV may circumvent this limitation.\textsuperscript{26} The use of cosmid, a special DNA construct that allows propagation of large pieces of DNA in bacteria, allows the entire HSV genome to be represented in overlapping fragments, thereby providing all the necessary function of a helper virus but without the risk of “recreating” a contaminant helper virus.

The early gene 1 (E1)-deleted adenovirus, another DNA virus with a respectable cloning capacity (8 kilobase [kb]), was an early viral vector developed for gene therapy. Deletion of the viral E1 gene rendered the virus replication deficient and created room for incorporation of a transgene. The virus is nonintegrating, its natural biology is well understood, and tools for constructing a recombinant virus are readily available.\textsuperscript{34} Figure 1 outlines the process involved in creating a recombinant first-generation adenovirus and its action at a target cell. Gene therapy for cystic fibrosis using adenovirus expressing the cystic fibrosis transport regulator protein was one of the earliest human clinical gene therapy trials attempted.\textsuperscript{35,36} Adenovirus vectors continue to be used in several human clinical protocols for cancer,\textsuperscript{37} cystic fibrosis,\textsuperscript{38} and monogenic liver disease.\textsuperscript{39} Early in vivo experience with adenovirus vectors in animals and humans revealed a fundamental limitation of the early-generation adenovirus vector for long-term applications: the viral infection elicits host cell and humoral immunity, resulting in elimination of the transduced cells. In the immunoprivileged CNS, adenovirus-mediated transgene expression can be detected as long as 2 months after infection,\textsuperscript{40} a respectable duration but still limiting for long-term gene therapy. Subsequent adenovirus incorporating a temperature-sensitive mutation in the viral single-stranded DNA-binding protein\textsuperscript{41} or constitutively expressing the viral gp19K protein\textsuperscript{42} were shown to result in longer host cell survival after viral transduction. These limitations of adenovirus vectors, in particular the
first-generation vectors, although serious for in vivo gene therapy applications, are not of major concern in experimental in vitro applications, where immune mediators do not exist. Subsequent elimination of viral E1 and E4 genes resulted in third- and fourth-generation adenovirus vectors with significantly less immunogenicity and increased payload capacity.\(^4\)\(^5\) Deletion of which of the multiple E3 and E4 gene products leads to better behaved virus remains unknown, but the question is largely supplanted by the new-generation “gutless” virus.

A recently described helper-dependent gutless adenovirus essentially devoid of all undesirable viral genome places us one step closer to the ideal viral vector. The gutless adenovirus is created by deletion of all viral genome except for the inverted terminal repeat and the &psi; sequence essential for viral packaging.\(^4\)\(^4\)\(^5\) The remaining viral DNA sequence in the gutless vector is minimal and similar to retrovirus or AAV. The space created by this deletion also expands the payload capacity of the virus, in theory, to more than 30 kilobases of DNA. Creation of a replication defective but infective gutless virus with minimal helper-virus contamination is dependent on use of the Cre-recombinase–expressing helper cell line.\(^4\)\(^5\)\(^6\) The technology depends on the property of the Cre-recombinase enzyme to selectively excise a segment of DNA flanked by a specific 30-base pair nucleotide called the lox P sequence. When a specially designed helper virus with its &psi; sequence flanked by lox P is used to create the helper-dependent gutless adenovirus in a cell line constitutively expressing Cre-recombinase, the excision of the &psi; sequence confers a selective disadvantage. The result is creation of the helper-dependent gutless adenovirus with minimal helper-virus contamination.

A study in mice and baboons demonstrates the superior in vivo longevity of the gutless virus in comparison to the first-generation adenovirus.\(^4\)\(^7\)\(^4\)\(^8\) No demonstrable vector-induced host immune response was seen. Although still a step removed from an ideal gene-delivery system because of the creation of a small amount of helper-adenovirus contamination during large-scale preparation (< 0.1%), this significant advance in adenovirus vector design clearly brings clinical application of viral vector-based gene therapy closer to reality.

**Obstacles in Viral Approaches.** Therapeutic intervention for pain requires gene delivery to CNS. Although viral vectors offer a promising approach to accomplishing this goal, there are numerous practical obstacles to realizing it. Delivery of most drugs to the CNS is hampered by the blood–brain barrier (BBB). Similar pharmacokinetic limitations pertain to gene therapy. The BBB is formed by tight junctions between capillary endothelial cells and excludes molecules greater than approximately 600 Da.\(^4\)\(^9\) Clearly, systemically injected viral vectors as well as naked oligonucleotide are excluded from effectively entering the CNS.\(^4\)\(^9\)\(^5\) Three methods are available for circumventing this limitation: (1) direct intraparenchymal or subarachnoid injection of compounds, (2) transient disruption of the BBB to allow permeation after systemic injection, or (3) entry into CNS through transport bypassing the BBB.

Direct deposit of viral particles into the subarachnoid space bypasses the anatomic limitations imposed by the BBB endothelial tight junction. However, a recent magnetic resonance imaging study of rat brains, in which the BBB was osmotically disrupted, indicates a barrier to cellular access imposed by basement membrane even in the absence of BBB. A similar barrier may prevent viral particles that have been directly deposited into the subarachnoid space from reaching spinal cord proper and thus limit the utility of adenovirus with approximately 90-nm diameter.\(^5\)\(^2\) In contrast, intrathecal injection of adenovirus resulted in a significant increase in β-galactosidase reporter expression in cells surrounding the subarachnoid space.\(^5\)\(^3\) Whether smaller virus such as the AAV with a 20-nm diameter can traverse this basement membrane barrier is not known. Direct intraparenchymal injection of adenovirus results in robust and reliable expression of transgene in the spinal cord proper.\(^5\)\(^2\)\(^\)\(^4\)\(^\)

Transient disruption of BBB allows systemically injected agents, including gene therapy viral vectors, to enter the brain parenchyma. Hyperosmotic mannitol disruption of the BBB, which has been used clinically to enhance the entry of chemotherapeutic agents into the brain, has been shown to enhance CNS entry of viral vectors as well.\(^5\)\(^5\)\(^−\)\(^5\)\(^7\) Alternatively, viral particles can be transported into the CNS after peripheral delivery by endogenous axonal transport system, thus bypassing the BBB. Natural entry of HSV into CNS via sensory nerve fibers after peripheral infection is known. Experimental transport of peripherally injected recombinant HSV and adenovirus has been demonstrated,\(^5\)\(^8\)\(^\)\(^9\) and a recent study demonstrated a long-lasting analgesic effect of peripherally injected and centrally transported HSV designed to express proenkephalin.\(^5\)\(^8\) In fact, selective transport and transduction of HSV into peripheral sensory ganglia and spinal cord neurons using axonal transport mechanisms may be ideal for targeted, anatomically restricted gene therapy for pain.

Direct viral injection into the subarachnoid space and expression of diffusible opioid peptide has been shown to provide analgesia.\(^6\)\(^\)\(^0\) Although virus and peptide production is limited to the subarachnoid space and excluded from spinal cord proper, β-endorphin peptides produced readily diffused unimpeded by the marginal glial cell anatomic barrier and showed expected naloxone-reversible decrease in pain sensitivity. The same concept can be extended to other nonopioid receptor targets where peptide agonists or antagonists exist and expand the possibility of viral vector-mediated gene therapy for pain through direct subarachnoid expression.

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of diffusible peptides. Figure 2 summarizes different avenues for delivery of therapeutic virus.

Two other issues limit the practical utility of all viral vectors currently available. The first is the inability to target the virus to a specific subset of cells, and the second is the host immune response. Currently, transduction of a limited number of target cells can only be attained through anatomically defined delivery of viral vectors. All available viral vectors are promiscuous, and all cells expressing the viral receptors that come in contact with the virus are transduced. One level of specificity can be attained by the incorporation of a cell type-specific promoter in the expression cassette. Promoters restrict transcription of the transgene only in selected cells that activate the promoter. Therefore, despite the prevalent viral transduction of many cells, expression of the transgene can be restricted. Examples of frequently used neuron-specific promoters include a 1.8-kb neuron-specific enolase promoter that directs expression in all neurons.61 A 1.1-kb 5′-untranslated region of dopamine β-hydroxylase directs expression of downstream gene in adrenergic and noradrenergic neurons.62 This promoter may be useful for anatomically defined expression of α2-adrenergic receptors for enhanced inhibition of synaptic neurotransmitter release. Ectopic but neuron-specific expression by a 500-base pair, γ-aminobutyric acid type A receptor, α6-subunit promoter has also been described.55 Expression of a transgene could be restricted to inhibitory neurons by the use of such a promoter. In general, it appears that 0.5–1 kb of 5′ untranslated sequence of many promoters is sufficient to confer expression of transgene, but a longer 3–4-kb regulatory sequence is necessary to confer neuron-specific expression in anatomically defined regions of the brain. A comprehensive table of neuron and glial gene promoters can be found in the report by Henson.64 However, in vitro specificity of promoters does not guarantee similar in vivo specificity, and caution must be exercised.25 The required size of the promoter precludes incorporation into most viral vectors, but payload size restriction is not a limitation for the gutless-adenovirus or cosmid-based HSV amplicons. Proper choice of promoter is essential for not only tissue-specific expression of the transgene but also long-term expression of the gene product. The frequently used constitutive viral promoters are downregulated in vivo, resulting in limited duration of transgene expression.65–67 Further expression regulation by an exogenous ligand (e.g., steroids, tetracycline, and rapamycin), in combination with a tissue-specific promoter will allow temporal regulation of transgene expression in addition to tissue specificity.48,68 Such temporal regulation of expression of antinociceptive gene product is particularly desirable because of the episodic nature of pain.

An alternative method to achieve target specificity is by coupling the viral particle to a target-specific peptide.69 An optimal short peptide with high affinity for the target tissue (airway epithelium in this study) was iden-
tified by bio-panning differentiated airway epithelium against a phage display library. The biologically selected high-affinity peptide was coupled to the viral particle using bifunctional polyethylene glycol molecule. The resulting peptide-coupled adenovirus with modified viral capsid demonstrated efficient transduction of the desired target cells in vitro. The target specificity of such a capsid-modified virus in vivo is not known at this time, but the polyethylene glycol coupling approach is attractive in that a smart virus can be targeted to any tissue by identifying the specific targeting peptide.

Early in the development of viral vectors, host immune response to vector administration was identified as a fundamental limitation to further exploration of viral vectors for in vivo gene therapy. Administration of viral vectors elicits a host immune response, which includes both cell and humoral immunity. The host immune response limits viability of virally transduced target cells and also precludes repeated administration of the same vector. Although the host immune response develops to most viral vectors, it is particularly problematic and therefore most thoroughly studied for the adenovirus. Removal of viral genes in the process of developing adenovirus vectors for in vivo gene therapy. Administration of viral vectors elicits a host immune response, which includes both cell and humoral immunity. The host immune response limits viability of virally transduced target cells and also precludes repeated administration of the same vector. Although the host immune response develops to most viral vectors, it is particularly problematic and therefore most thoroughly studied for the adenovirus.

Advantages of Oligonucleotide Therapy. The promise of antisense therapy is the ability to inhibit expression of a specific gene. Conventional pharmacologic agents achieve their actions through proteins, are not entirely selective, and may show many side effects, including receptor upregulation. Current conventional drugs cannot act at specific intracellular proteins. Because of the specificity of antisense therapy, rational antisense oligonucleotide drug synthesis and design may be much easier than current agents. Figure 3 summarizes how oligonucleotide may interfere with normal gene activity, resulting in a decrease in target protein expression, termed protein knockdown. In addition, antisense drugs may be used for a variety of disorders. The greatest advantage, however, is the significant amount of clinical experience with oligonucleotide-based drugs.

Cumulative experience with in vivo oligonucleotide studies over the last decade has defined the fundamental bioavailability and pharmacokinetic properties of oligonucleotides as well as unmasked unexpected problems of toxicity. Oligonucleotides are efficiently removed by the reticuloendothelial system when injected intravenously, subcutaneously, or intraperitoneally. After intravenous administration, radioactivity-tagged short oligonucleotides show a typical distribution half-life (T1/2a) on the order of 1 h and an elimination half-life (T1/2b) of 40–50 h independent of the actual sequence. The major route of elimination is through urine, although some oligonucleotides are excreted in feces. Recent data indicate that oligonucleotide is systemically absorbed after oral administration, leading to the possibility of an oral regimen for oligonucleotide drugs. Long-term toxicity of oligonucleotides, in particular with the phosphorothioate modification, include dose-dependent but reversible lymphoid hyperplasia, splenomegaly, and multior-

Antisense Oligonucleotides

The second phase of the immune response is caused by expression of viral proteins. Although replication-deficient E1-deleted adenovirus was engineered to limit expression of viral proteins, low-level expression of viral hexon and penton (proteins that form the viral capsid), fiber proteins (protein that forms the antennae-like projections off the capsid), and other late gene products activate host cytotoxic T lymphocytes. The cell-mediated immune response largely limits the viability of virus-transduced cells during the initial virus administration. The fundamental design of MMLV and AAV vectors and, more recently, the gutless adenovirus, essentially devoid of all viral genes, circumvents problems associated with basal expression of viral proteins. A third-phase humoral response to cleared viral particles results in antiadenovirus antibody production. The humoral response limits effectiveness of subsequent adenovirus administration. Both the cytotoxic and humoral responses to adenovirus are conventional in requiring interactions with class I and II major histocompatibility complex molecules. Transient immnosuppression of the host with coadministration of anti-CD4 antibody, cyclophosphamide, or FK506 have been shown to prolong the duration of transgene expression. Sequential administration of adenovirus of different serotypes circumvents immunemediated inactivation, suggesting the real possibility of attaining long-term expression of transgene.

Although incapacitated to some degree, currently available viral vectors cannot be considered as risk-free. As we gain further insight into the nature of host immune response to viral vectors and as better vectors are developed, long-term and repeated administration of therapeutic viral vectors should become a reality. Currently available viral vectors are not ready for use in therapeutic intervention, but the technology is ready for experimental studies and limited human clinical trials. The gutless adenovirus and cosmid-based HSV amphiol vectors offer the greatest promise toward achieving an ideal viral vector for human gene therapy.
In addition to these toxicities resulting from presumed immune system stimulation, prolongation of coagulation profile most likely caused by specific interactions between oligonucleotide and thrombin, has been reported.87 Overall, reports of systemic toxicity even after prolonged oligonucleotide administration are few, and several human clinical trails with antisense oligonucleotide are in progress.88–90 At least one antisense oligonucleotide designed to treat cytomegalovirus-induced retinitis (ISIS 2922) has successfully a completed phase III clinical trial and has just been released for marketing. Another antisense oligonucleotide targeting the HIV gag gene (GEM 91) has undergone extensive phase I–II human clinical trials and is nearing a full-scale phase III trial (http://www.hybridon.com). This antisense oligonucleotide decreases the production of p24 protein in HIV-infected lymphocytes to a degree comparable to the antiviral drug zidovudine, which is the currently accepted standard of antiviral drug treatment.91 There is no doubt that systemic administration of oligonucleotide is well tolerated in humans when administered at the proper dose, and this class of gene-targeted drug will enter the clinical armamentarium before long.

In contrast to the numerous human clinical trials of systemic oligonucleotide administration, there are no reports of direct CNS administration of oligonucleotides after systemic routes of administration. This pharmacokinetic problem can be circumvented by direct injection of oligonucleotide within the CNS. Intraparenchymal, intracerebral ventricular, and intrathecal routes either by a single injection or by implantation of an intrathecal catheter for long-term administration have been reported in animals. Unlike viral particles, marginal glial cells do not limit diffusion of oligonucleotide from the intrathecal space to spinal cord proper. Successfully targeted genes with biochemical and functional evidence of protein knockdown after intraparenchymal or intracerebral ventricular injections include the neuropeptide Y receptors,92 brain calcineurin,93 N-methyl-D-aspartate receptor NR1 subunit,94–97 brain c-fos,98,99 and brain endothelial nitric oxide synthase.100 Many of these CNS targets already shown to be sensitive to antisense therapy also play a significant role in pain. No behavioral abnormalities suggestive of systemic or nonspecific toxicity of the oligonucleotide administration were noted. The intrathecal route of oligonucleotide administration has been used for targeting c-fos,101 µ-opioid receptor,102 δ-opioid receptor,103 neurokinin-1 receptor,104 and gene 2/IP-10 in an experimental model of allergic encephalomyelitis.105 In this study, Wojcik et al. reported hind-limb paralysis and tissue necrosis of the spinal cord after intrathecal infusion of phosphorothioate but not phosphodiester oligonucleotides, raising the possibility of toxic effects of the back-bone–modified

Fig. 3. Antisense oligonucleotide and potential sites of action. (A) Normal gene activity (DNA to messenger RNA [mRNA] to protein) results in production of a pronociceptive protein mediating pain (left). Oligonucleotide enters the cell and hybridizes to the complimentary mRNA target. This prevents production of the protein, resulting in a selective protein knockdown (right). (B) Oligonucleotide (short dashed or solid bars) may act at multiple sites between gene transcription and final protein targeting.
nucleotides. However, a specific interaction between the systemic administration of myelin basic protein leading to experimental allergic encephalomyelitis and the intrathecally administered phosphorothioate oligonucleotide cannot be ruled out. Work in our laboratory demonstrated no overt toxicity in rats treated with thiolated antisense oligonucleotide targeting the N-methyl-D-aspartate receptor NR1 subunit.

A recent pharmacokinetic investigation of brain intraparenchymal infusion of [35S]-labeled 18-mer oligonucleotide indicates extensive spread of the oligonucleotide throughout the brain and cerebral spinal fluid. Postinfusion radioactivity decayed over the next 12 h with a monoexponential time course, with a time constant of 3 h (approximated from fig. 5 in the report by Broaddus et al.106). Comparable data for intrathecal administration do not exist, but similar pharmacokinetic properties or a more rapid and diffuse distribution can be expected after a direct deposit of oligonucleotides within the cerebral spinal fluid. Observations documenting successful functional alteration of many different gene products and the desirable pharmacokinetic properties in the CNS are particularly encouraging when considering antisense oligonucleotide as a potential therapeutic drug in humans. The intrathecal route of delivery as a single injection or through an infusion catheter is in active clinical practice today for CNS administration of a variety of drugs. The relative noninvasive nature, ability to deposit oligonucleotides directly near the site of nociceptive processing in the spinal cord, and vast clinical experience with the intrathecal route of administration makes this a very attractive method for antisense oligonucleotide delivery.

Unlike viral vectors, antisense oligonucleotides have minimal immunogenic properties, do not produce potentially toxic viral proteins, and are effective in nondividing cells. In addition, antisense oligonucleotides are not integrated into the genomic DNA, thus virtually eliminating the possibility of genomic alterations. Titration to an acute biologic effect, such as nociception, may be easier with antisense oligonucleotide because of its shorter duration of action.

**Obstacles in Antisense Therapy.** Although the concept of antisense oligonucleotide inhibition of a target RNA seems simple, the reality of antisense oligonucleotide isolation and application has been much more difficult than originally anticipated. Investigators have encountered many difficulties, including generation of active oligomers, specificity of antisense oligonucleotides, access of antisense oligonucleotides to target RNAs, and occurrence of unexpected non-antisense effects. Despite the promise that antisense oligonucleotides can exclusively target one specific gene to prevent expression of the relevant protein, no one has actually proven that antisense oligonucleotides can eliminate expression of a single gene.107

**Isolating an Appropriate Oligomer.** At the present time, there are no blueprints to guide an investigator in generating active oligomers. The current process is essentially a random selection of complementary oligomers (each corresponding to different sites) to the cDNA or mRNA of interest. For each active oligomer that is obtained, approximately seven or eight oligomers must be screened. Individual testing of each active oligomer must be undertaken to determine the oligomer with the greatest specificity and lowest inhibitory concentration. Although the number of active oligomers that should be screened to find one that is maximally active is not known, one expert has suggested that it would be optimal (although costly) to screen approximately 30–40 active oligomers. Only 3% of antisense oligonucleotides may be highly effective. Thus, present methods for selection of an oligomer that is maximally active from a large pool of candidates is an extremely time-intensive process. Rational design of effective antisense oligonucleotide may reduce the number of oligomers that need to be tested in vitro and in vivo. Potential methods include RNase H mapping of target mRNA (to identify accessible sites for antisense oligonucleotides) and scanning combinatorial oligonucleotide array (to identify all possible overlapping antisense oligonucleotides of every length up to a given length).

**Specificity and Discrimination of Oligonucleotide.** Oligomer length is an important factor in determining target site recognition for antisense oligonucleotide. Although it would seem that choosing an oligomer with a longer sequence of nucleotides would confer increased specificity to target mRNA and discrimination from other sites with similar nucleotide sequences, increasing the oligomer length beyond approximately 10 nucleotides may actually have the opposite effect of stabilizing antisense oligonucleotide binding to mismatched sequences. For example, an oligomer with a sequence of 13–17 nucleotides, which most likely would be unique, may bind not only to target RNA but also bystander RNAs possessing one or two nucleotide mismatches. In other words, there are potentially 209 and 12 alternate matches for a 13-nucleotide antisense oligonucleotide if 2 and 1, respectively, base mismatches occur. In addition, increasing antisense oligonucleotide length may not increase specificity as RNase most likely requires only a short sequence (7–10 nucleotides) to cleave the RNA strand of an mRNA-antisense oligonucleotide complex. Unfortunately, using a shorter nucleotide sequence will increase the probability that other nontargeted RNAs will have a similar base sequence and be bound by antisense oligonucleotide with subsequent cleavage by RNase. Despite the theoretical limits of antisense specificity and discrimination, there seems to be a general consensus that the...
optimum length for an antisense oligomer is between 18 and 20 bases.\textsuperscript{108}

**Intracellular Access and Binding of Oligonucleotide.** Effects produced by antisense oligonucleotide \textit{in vitro} may not translate into similar effects \textit{in vivo}, partly resulting from barriers to intracellular access and RNA binding. Unlike the artificial situation inside cells where antisense oligonucleotides bind to naked RNAs, antisense oligonucleotides used in clinical situations must proceed intact to the target cell, obtain intracellular access into the cytoplasm and nucleus, and conform with and securely bind to target RNA to allow cleavage by RNase. Oligonucleotides are rapidly absorbed and widely distributed after parenteral (e.g., intravenous, intradermal) administration; however, there have been no data that suggest significant BBB penetration occurs after systemic oligonucleotide administration.\textsuperscript{80} When given intraventricularly or intrathecally, antisense oligonucleotides, unlike viral vectors, do not appear to have difficulty transversing pial membranes and diffusing into neurons.\textsuperscript{113,114} Intraventricular injection of antisense oligonucleotides has been shown to decrease intraneuronal target mRNA and protein expression.\textsuperscript{94,115}

Factors that determine cellular uptake of oligonucleotides are not clear; however, oligonucleotides are broadly distributed intracellularly once cellular uptake occurs.\textsuperscript{83} If oligonucleotides do not bind to nontargeted proteins or become entrapped in intracellular endosomes or lysosomes, they then have the possibility to bind to target RNA, a potentially difficult proposition in itself.\textsuperscript{116} \textit{In vivo} RNA is a complex three-dimensional structure, and targeted areas may not be accessible or protected by cellular proteins or ribonucleoprotein complexes that prevent antisense oligonucleotide binding or ribozyme-mediated cleavage.\textsuperscript{107,117} Thus, RNA structure is a major determinant of antisense oligonucleotide access and eventual binding \textit{in vivo}.

**Non-antisense Effects.** One of the most significant problems in the use of antisense oligonucleotides is the wide variety of unexpected non-antisense effects that may occur.\textsuperscript{107} Because of the nature of oligonucleotide specificity (see Specificity and Discrimination of Oligonucleotide), antisense oligonucleotides most likely also bind to nontargeted RNAs, thus potentially causing many unpredictable side effects clinically, some of which actually may be potentially very useful.\textsuperscript{120} In addition, certain combinations of nucleotides (e.g., four continuous guanosine residues) are known to inhibit protein expression in a sequence-independent fashion.\textsuperscript{121} The investigator may not be certain whether the desired effect (if it occurs) is a result of antisense oligonucleotide reaction with the intended target or a nontargeted protein. Non-antisense effects may occur through totally unexpected mechanisms with elucidation of exact mechanisms of action, potentially taking years to determine.\textsuperscript{105}

**Toxicity.** Oligonucleotides may show sequence-independent and -dependent toxicity. Sequence-independent effects, including immune stimulation, thrombocytopenia, hepatic transaminase elevation, complement activation, and prolongation in clotting times, may be caused by the polyanionic character of phosphorothioate oligonucleotides.\textsuperscript{122} Phosphorothioate molecules may interact nonspecifically with cellular targets, resulting in extensive cellular toxicity.

Sequence-dependent toxicity is said to occur when administration of phosphorothioate oligonucleotide of various lengths results in comparable toxicity profiles of varying severity.\textsuperscript{122} Oligonucleotide toxicity may be a limiting factor in therapeutic use of antisense oligonucleotides as steep dose–response curves and narrow therapeutic windows (only a factor of 10 may differentiate the concentration producing no effect from that generating a full effect) may be present \textit{in vivo}.\textsuperscript{107}

**Interpretation of Data from Antisense Oligonucleotide Studies.** One of the most difficult problems in interpreting data from antisense oligonucleotide studies is determining whether the observed effect is produced by target antisense interaction or other nontargeted mechanisms. As previously described, many non-antisense effects may occur and interfere with data obtained \textit{in vivo}. Proof of an antisense mechanism \textit{in vitro} and \textit{in vivo} will require an adequate number of controls, direct measurement of target protein or RNA, full dose–response curves with rank order potencies of analogs and mismatches, lack of effect on closely related gene products and housekeeping genes, and explanation of unexpected effects produced by control oligonucleotides.\textsuperscript{121,125} The field of antisense drug development is still in its infancy, and proposed experimental guidelines will increase pharmacokinetic, pharmacologic, and toxicologic knowledge to ultimately use antisense oligonucleotides for therapeutic purposes.\textsuperscript{108,121,125}

**Conclusions**

Gene therapy offers the tantalizing possibility of specific and selective targeting of a single point in the synthesis of proteins. Recently, the fields of molecular and cellular biology have seen significant advances in techniques and tools available for gene therapy. Current gene therapy tools are promising but still require significant improvement before routine human clinical application becomes a reality. CNS delivery of antisense oligonucleotides as a means for reducing the target protein level is a technology on the verge of human clinical trials, and viral vectors clearly will improve as the entire field of human gene therapy evolves. Animal work with state-of-the-art viral vectors conclusively demonstrates the feasibility of tissue-specific and regulatable expression of transgenes. Application of the tools and strategies
of gene therapy described here to the field of pain medicine may yield valuable therapies for the management of pain resistant to conventional pharmacotherapeutic options. In Part II of this review, selected potential targets for gene therapy for the management of pain will be presented. Each putative target will be reviewed for evidence of its role in nociception, molecular biology, and receptor function. Most importantly, specific studies using a gene therapeutic approach to the study of nociception will be summarized.

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