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Retrovirus Vectors and Their Uses in Molecular Biology

Eli Gilboa

Summary

Retroviral vectors utilize the biochemical processes unique to retroviruses, to transfer genes with high efficiency into a wide variety of cell types in tissue culture and in living animals. With such vectors, the effect of newly introduced genes and the mechanism of gene expression can be studied in cell types so far refractory to other methods of transfer.

Introduction

The most widely used method of introducing genes in a genetically stable manner into mammalian cells is DNA transfection,¹ in which purified DNA is

co-precipitated with calcium phosphate or dextran sulfate and added directly to the cells. At low frequency, viable cells can be isolated which contain one to several copies of the transfected DNA integrated into the chromosome. DNA transfection has several drawbacks that severely limit its use as a general method for introducing genes into mammalian cells. First, it is very inefficient. At best, only a small fraction of cells will have incorporated the newly transferred gene. Secondly, only a small number of *in vitro* cultured cell lines are susceptible to this low level of gene transfer. In the past several years, the use of novel gene transfer techniques has been investi-

gated. Protoplast fusion² and electroporation³ exhibit a broader host range of susceptible cells, enabling the transfer of genes into many lymphoid cell lines. However, the efficiency of gene transfer is still very low. A third technique which shows much promise utilizes a virus to deliver genes into recipient cells and is the subject of this review.

Biology of Retroviruses

Retroviruses are RNA viruses; the viral genes are encoded in a single-stranded RNA molecule. After the virus penetrates into the cell, the viral RNA is

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converted to a double-stranded DNA molecule (this process is called reverse transcription). This DNA enters the nucleus and integrates into the cell chromosome, becoming indistinguishable from any other cellular gene. The integrated viral DNA form, called a *provirus*, is the template for the expression of the viral genes and the synthesis of the progeny virion RNA. The viral gene products and progeny RNA assemble into a virion which leaves the cell by budding through the outer membrane. It is important to point out that the integration of the viral genome into the cell chromosome is a normal part of the viral replication process which is mediated by virally encoded functions. With a few exceptions, the presence of the viral genome in the cell, expression of its genes, and the formation of progeny virus have no apparent effect on the viability of the infected cell. The cell is chronically infected, but otherwise healthy, and continuously secretes virus into the medium.

Principles of Retrovectorology

The suitability of retroviruses for gene transfer stems from their mode of replication. By 'simply' replacing the viral genes with the gene of interest and utilizing the efficient viral infection process, the gene is transferred into the target cell as if it were a viral gene. Fig. 1 is a schematic diagram showing how it works. First, a retrovirus carrying the foreign gene is produced, and next, the hybrid or recombinant virus is used to infect the target cell. Generation of a recombinant retrovirus proceeds in two steps. First, using standard recombinant DNA techniques, portions of the viral DNA are combined with the gene of interest. As shown in Fig. 1, most of the internal viral sequences may be replaced with the foreign gene. The remaining retroviral DNA, called the *vector*, always includes the two ends of the viral genome, which are terminally redundant and are called long terminal repeats (LTRs). This and immediately adjacent regions of the viral genome contains important (*cis*) functions necessary for the replication of the virus. The deleted sequences, which may be replaced with the foreign gene, encode viral proteins that are necessary for the formation of infectious virions (*trans* functions). These proteins, also necessary for the replication of the virus, can be complemented *in trans* if the cell contains another virus expressing the gene products missing in the vector. In the second step, the hybrid DNA is in-

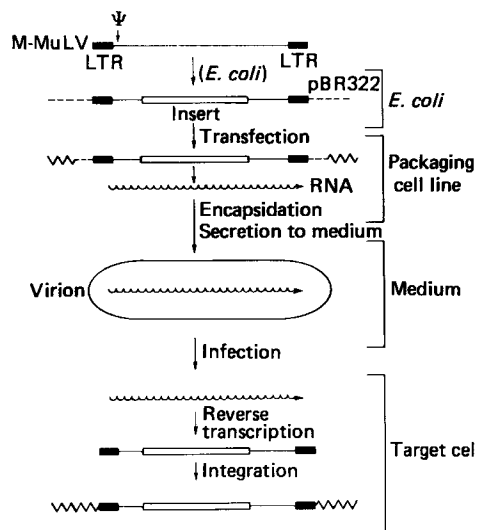


Fig. 1. Use of retroviral vectors to introduce genes into eukaryotic cells. Moloney murine leukemia virus (*M-MuLV*) is a prototype murine retrovirus used in many studies as a source of retroviral vectors. Ψ , Packaging signal, directs the incorporation of the retroviral transcript into virions. See text for more details.

troduced into specially designed *in vitro* cultured cells by standard (and inefficient) DNA transfection procedures. These cells, called packaging cells,⁴ harbor a retrovirus defective in a *cis* (Ψ) function. Its RNA cannot encapsidate into a virion but it does express all the viral proteins (*trans* functions) and is therefore able to complement the same functions missing in the incoming vector DNA. The vector DNA is now transcribed into a corresponding RNA which is encapsidated into a retrovirus virion and is secreted into the medium. The actual gene transfer starts at this point: the virus collected in the medium is used to *infect* the target cells, and through the efficient viral infection process the foreign gene is inserted into the cell chromosome as if it were a viral gene (for further reading, see ref. 5).

Why is retroviral based gene transfer such a promising technique? Mainly for two reasons. First, because of its potential high efficiency, it is the only system available for use in cases where it is necessary for the gene of interest to be introduced into a large proportion of the target cells. This is in sharp contrast to other gene transfer systems such as DNA transfection,¹ protoplast fusion² and electroporation.³ Secondly, retroviral vectors have a broad host range, enabling the introduction of genes not only into monolayer-grown cells such as NIH 3T3 or L cells but also into many suspension-grown lymphoid and myeloid cells, and hemopoietic stem

cells present in the bone marrow population.

As with many new emerging technologies, the potential of retroviral gene transfer created great expectations. However, it was soon realized that there are certain limitations and difficulties associated with this technology. The basic limitation of using retroviral vectors compared to alternative types of gene transfer techniques is that it requires extra manipulations, and is thus more time-consuming. When using DNA transfection, electroporation or protoplast fusion, the DNA fragment carrying the gene of interest is directly introduced into the target cells, whereas using retroviral vectors the gene of interest is first inserted into a retrovirus vector and converted into a virion before the actual gene transfer takes place (Fig. 1).

The use of retrovirus vectors has encountered an even more serious difficulty. With numerous refinements, it is now quite simple to insert a gene into a retrovirus vector, obtain recombinant virus, infect target cells and express the foreign gene. What is however more difficult and elusive at this moment is to optimize the *efficiency* of the process. Two parameters determine the efficiency of retroviral vectors. The first is the ability to infect a large proportion of the target cells. This property is a function of the titer of recombinant virus that can be produced in the process as described in Fig. 1. Secondly, once delivered into the target cells the gene has to be expressed properly.

The nature of the retrovirus vector will determine to a large extent both parameters. The reason why the development of an all-purpose super-efficient retrovirus vector is elusive stems from the simple fact that we don't understand yet some of the more subtle details of the structure and biology of retroviruses nor that of mammalian genes. Most often difficulties arise when the hybrid virus is constructed by replacing viral genes with a foreign gene (Fig. 1). The removal of viral sequences and substitution of foreign DNA can cause a substantial reduction in the titer of viruses generated and can also reduce the efficiency with which the transduced gene is expressed. Since different substitutions can create variable negative effects and the requirements for optimal foreign gene expression are largely unknown, the outcome of any particular combination cannot be predicted.

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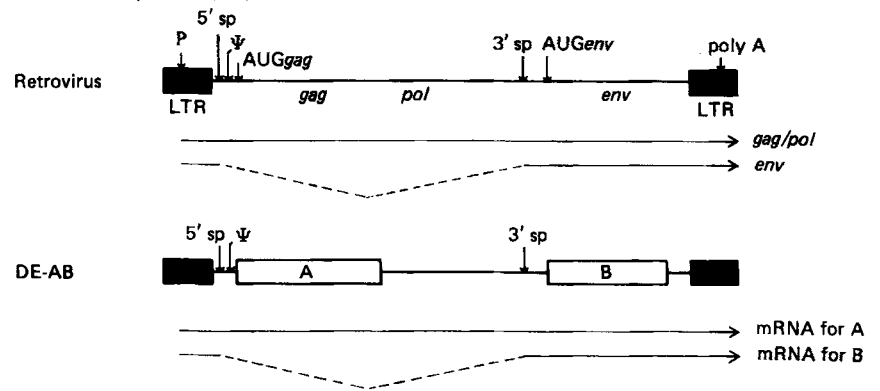
Retrovirus Vectors

Three types of retroviral vector are shown in Fig. 2. Note that these vectors accommodate not one but two genes. One gene is the gene of interest and the second gene is a *selectable* gene. A selectable gene is not absolutely required, but its presence greatly facilitates the use of retroviral vectors, enabling the identification and isolation of productively infected cells. In some cases, the presence of a selectable gene may have negative effects, and the use of vectors without selectable genes, although more cumbersome, may be considered.

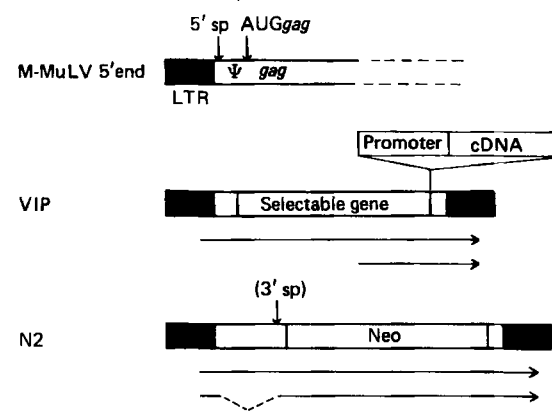
Fig. 2A shows the structure of a prototype double expression (DE) vector as described by Cepko *et al.*⁶ As shown in Fig. 2A (top), normal viral genes are expressed from two RNA species. The *gag* and *pol* genes are expressed from an unspliced RNA form which is co-linear with the viral genome and the *env* gene is expressed from a spliced RNA form, produced from the unspliced RNA species by the removal of a long intron. Removal of the viral intron is tightly regulated in this system, since both RNA species, the spliced form and its precursor, the unspliced form, accumulate in the cytoplasm in optimal proportions. DE vectors (Fig. 2A, bottom) contain two foreign genes. One gene, replacing the *gag/pol* segment, is expressed from the unspliced RNA form; and the second gene, replacing the viral *env* gene, is expressed from the spliced RNA form. The distinguishing feature of this type of vector is that it provides not only the (*cis*) functions necessary for the transmission of the foreign genes into the target cells but also provides the (*cis*) functions for their expression, such as an enhancer, a promoter, and a 5' splice site present in the 5' LTR and downstream sequences, a polyA signal in the 3' LTR, and a 3' splice site encoded in a third DNA fragment.

DE vectors are dependent on the efficient formation of the viral RNA species. This in turn depends on a properly regulated splicing process, and the underlying assumption in the design of these vectors⁶ was that removal of the viral intron is regulated by the sequences immediately surrounding the splice junctions. There is now mounting evidence that this assumption is not correct. Instead, sequences scattered throughout the viral intron were found to play an essential role in modulating the levels of spliced and unspliced

(A) Double expression (DE) vectors



(B) Vectors with internal promoters (VIP)



(C) Self-inactivating (SIN) vectors

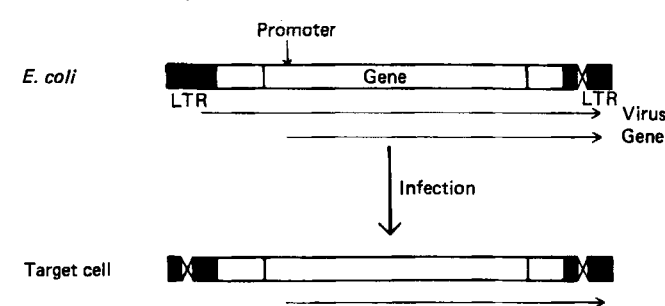


Fig. 2. Three strategies of retroviral vector design. See text for details.

RNA forms that accumulate in the cytoplasm.^{7,8} Thus the absence of intron-contained sequences in DE vectors may be one reason for their poor performance. A second and more important limitation in using DE vectors is inherent in their structure. In DE vectors the expression of the gene of interest is directed from the promoter encoded in the LTR, and therefore the usefulness of these vectors will be limited to cells in which the viral promoter is active.

Fig. 2B shows the structure of another type of retroviral vector in which the transduced gene is expressed from an internal promoter, hence the name, vectors with internal promoters

(VIP). In these vectors, the selectable gene is linked to the left end of the viral DNA and is expressed from the viral promoter.^{9,10} A 'minigene' consisting of a DNA fragment encoding a promoter linked to a cDNA copy of the gene of interest⁹ is inserted downstream of the selectable gene as shown in Fig. 2B. The promoter-encoding DNA fragment, which is responsible for the expression of the transduced gene, can be derived from any gene and therefore, in using this type of vector, one has the flexibility of choosing the promoters to express the transduced gene in a manner most appropriate for a particular experimental design. The main drawback of this strategy of vector design is

that the presence of a promoter internal to the viral vector may affect critical vector functions, i.e. the titer of viruses generated, and viral promoter activities.^{10, 11, 12}

Fig. 2B (bottom), shows the structure of a VIP vector called N2, which differs from similarly constructed vectors. In addition to the LTRs, retroviral vectors must contain a region downstream of the 5' LTR which encodes the viral packaging signal (Ψ). This region excludes the *gag* AUG initiation codon so that the gene fused downstream of these sequences will be translated properly. As shown in Fig. 2B (bottom), in the N2 vector the region downstream of the 5' LTR extends beyond the *gag* AUG initiation codon and includes 418 base pairs of the *gag* coding sequences to which the bacterial Neo gene is fused. It appears that this extra region present in N2 is responsible for the production of 10- to 50-fold higher titers of virus as compared to similar vectors lacking these sequences. In N2 the functional AUG and 418 base pairs of *gag* coding sequence, are out of frame with the Neo gene coding sequences. How is the Neo gene expressed? As illustrated in Fig. 2B, it appears that a cryptic 3' splice site is activated in the *gag* coding sequences upstream of the Neo gene, generating a spliced RNA form which serves as the mRNA for the Neo gene (Armentano *et al.*, in preparation). Reports from several laboratories suggest that the N2 vector and its derivatives are very useful for the transfer of genes into suspension-grown lymphoid cells and especially into bone marrow progenitor cells of various species.

Self-inactivating (SIN) Vectors

SIN vectors are the latest addition in retrovirus vectors, and they have a very interesting property.¹³ The LTRs at the two ends of the retroviral genome contain an element called an *enhancer* which can affect the expression of the foreign gene in VIP vectors and which can activate adjacent oncogenes when integrated into the cell chromosome. This is a worrisome aspect if retroviral vectors are to be used in human therapy. The special property of the SIN vector is, as its full name implies, that upon integration into the chromosome of the target cells it self-inactivates because a small portion of the viral DNA, which includes the enhancer and promoter sequences, is absent from both LTRs. Consequently, the proviral DNA in the infected cells becomes

transcriptionally inactive, enabling the uninhibited expression of the foreign gene. In addition, the absence of the viral enhancers will greatly diminish the possibility of activating cellular oncogenes. Fig. 2C shows how this works. SIN vectors contain a small deletion in the 3' LTR which encompasses the promoter and enhancer sequences that control the accurate and efficient transcription of the viral genome. These sequences are required for viral gene expression when present in the 5' LTR, but not in the 3' LTR. Therefore, their removal from DNA constructs as shown in Fig. 2C, does not affect viral functions. As a consequence of the replication of retroviruses (see review in ref. 14), a region of the 3' LTR encompassing this deletion, called the U3 region, is the template for the synthesis of the U3 regions in both the 5' and 3' LTRs in the next generation. Thus the deletion encompassing the viral enhancer and promoter will be transferred to both LTRs in the target cells.

Although it has been demonstrated that SIN vectors do self-inactivate in the target cell, the titers of virus generated from this type of vector are disappointingly low (10^3 – 10^4 cfu/ml).¹³ and probably not sufficient for use in applications involving *in vivo* gene transfer. It is hoped that appropriate modifications, together with advances in the understanding of the retroviral genome, will increase the titer and performance of this type of vector as well.

Uses of Retroviral Vectors

Have retrovirus vectors fulfilled their promise or will they do so in the future? Specifically stated, have retroviral vectors been used in studies which yielded significant findings? The answer is yes, and this is illustrated in two examples which demonstrate the usefulness of retroviral gene transfer to study gene expression in suspension-grown lymphoid and myeloid cell lines, many of which are refractory to alternative gene transfer techniques such as DNA transfection. A third example will describe the use of retrovirus vectors in hemopoietic lineage analysis.

Lang *et al.*¹⁵ have tested the hypothesis that autocrine growth stimulation, often exhibited by tumor cells, contributed to the process of malignant transformation. The experimental approach was as follows. FD cells are a continuous hemopoietic cell line with

some characteristics of myeloid progenitor cells which do not express the endogenous GM-CSF gene. These cells are absolutely dependent on GM-CSF, a growth factor essential for both survival and proliferation. FD cells are non-tumorigenic when injected into mice. In order to test the effect of constitutive expression of the growth factor, Lang *et al.*¹⁵ have introduced into FD cells the gene for GM-CSF under the control of the retroviral LTR using a DE vector. The vector-infected cells which synthesized and secreted GM-CSF grew independently of exogenous growth factor and, most strikingly, produced tumors in syngeneic mice. Thus experimentally induced autocrine growth regulation in a factor-dependent cell line resulted in tumorigenicity. Clearly, the use of a retrovirus vector was necessary to introduce and express the GM-CSF gene in the suspension-grown hemopoietic cell line.

A second example illustrating the usefulness of retroviral vectors is provided by the studies of Dayton *et al.*¹⁶ showing that the HTLV-III/LAV transactivator (*tat*) gene product is required for the replication of the virus. First, it was shown that viruses containing deletions spanning the *tat* gene sequences are incapable of replication and do not demonstrate the cytopathic effects characteristic of this virus. In order to determine whether the *tat* gene function is indeed responsible for the observed defect, a complementation experiment was performed. The virus carrying the deletion in the *tat* gene was introduced into modified Jurkat cells (a human T helper cell line) which constitutively expressed the *tat* gene product. It was shown that the *tat* gene product present in the Jurkat cells effectively complemented the defect in the deleted virus. Jurkat cells which expressed the *tat* gene product constitutively were derived by infection with a recombinant retrovirus carrying the *tat* gene. The establishment of permanent cell lines expressing the *tat* gene, as opposed to similar studies in which the *tat* gene was transiently expressed in cells by using DNA transfection procedures,¹⁷ demonstrated the advantages of using retroviral vectors.

In Vivo Gene Transfer

The special features of retroviral gene transfer have provided for the first time the opportunity of introducing genes into the somatic cells of living individuals. Although this technique is at

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present limited to gene transfer into hemopoietic cells, its potential in general studies and applications to human therapy is well recognized. Basically, the technique, as applied to mice, involves the removal of bone marrow cells from an individual mouse, infection *in vitro* with a recombinant retrovirus, and transfer of the infected bone marrow cells back into a lethally irradiated recipient mouse. Irradiation is used to eliminate the hemopoietic cells of the recipient mouse. This mouse is rescued from death by the donor cells, which repopulate its hemopoietic system. If a hemopoietic stem cell or committed progenitor cell present in the donor bone marrow cell population was infected with a recombinant retrovirus, all its descendants (the mature blood cells) will contain the vector and will hopefully also express the transduced gene.

Hemopoietic Lineage Analysis

In this example, retrovirus vectors were used to study the lineage relationships within the hemopoietic stem cell hierarchy. Since integration of retroviruses into the cell chromosome is apparently random, the integration sites can be used as unique clonal markers to identify hemopoietic precursors and to follow their progeny through differentiation. Experimentally, the unique integration sites are identified by restriction analysis, DNA blotting and hybridization with a vector-specific probe. Retrovirus vectors are used in these studies rather than wild-type virus for technical reasons—the viral sequences cross-hybridize with many cellular sequences which are closely related to endogenous viruses. In a retroviral vector, the transduced gene provides a unique hybridization probe. Bone marrow cells infected with retroviral vectors are used for long-term reconstitution of the hemopoietic system of recipient mice, and the unique integration sites are examined in mature cells of various lineages. Using this approach, it was demonstrated in several independent studies^{18, 19, 20} that the same integration site was present in mature hemopoietic cells of both myeloid and lymphoid lineage. It follows that a common precursor was infected with a retroviral vector and gave rise to both myeloid and lymphoid cells. Although the concept of a common precursor for the lymphoid and myeloid lineages was already supported by several lines of study, these results provide direct evidence for the presence of a primitive

pluripotent hemopoietic stem cell.

The three examples cited above demonstrate unequivocally the usefulness of retroviral gene transfer in biological studies in diverse fields such as oncogenesis, virology and hematopoiesis. The examples so far are few, but it is expected that the list will grow fast. Probably one of the more significant impacts of this new technology in the future stems from the capability of introducing genes into hemopoietic cells of adult individuals. Transfer of genes and analysis of their effects in the live animal will undoubtedly provide new and exciting insights.

The use of retroviral vectors also holds promise for human therapy. The ability to transfer genes into live individuals is the technical basis for developing an effective somatic gene therapy procedure to treat genetic disorders of the hemopoietic system,²¹ and it appears now that retroviral vectors will play a major role in this process. Gene transfer in live animals is now in its infancy. Efficiency of gene transfer is low, and problems regarding the expression of the transduced gene have yet to be solved. Unfortunately, it is not possible to predict whether these are short-term or long-term problems awaiting solution.

In summary, although some retroviral vectors were shown to be highly effective in various studies, it is not possible so far to recommend one particular vector for general use. Problems with stability of the recombinant vector, titer of virus and expression of the transduced genes still exist. The use of retrovirus vectors should be approached with caution, and it is advisable that several alternative strategies be explored in parallel.

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Coronavirus Leader-RNA-Primed Transcription: an Alternative Mechanism to RNA Splicing

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Summary

Many viral and cellular mRNA species contain a leader sequence derived from a distant upstream site on the same gene by a process of RNA splicing. This process usually involves either nuclear functions or self-splicing of RNA molecules. Coronavirus, a cytoplasmic RNA virus, unfolds yet another mechanism of joining RNA, which involves the use of a free leader RNA molecule. This molecule is synthesized and dissociates from the template RNA, and subsequently reassociates with the template RNA at downstream initiation sites of subgenomic mRNAs to serve as the primer for transcription. This leader-primed transcriptional process thus generates viral mRNAs with a fused leader sequence. A similar mechanism might also operate in the mRNA transcription of African trypanosomes.

Introduction

Coronaviruses are a newly recognized group of viruses which infect many animal species, causing a variety of respiratory and gastrointestinal illnesses. Among these viruses are porcine transmissible gastroenteritis virus (TGEV), bovine coronavirus (BCV), avian infectious bronchitis virus (IBV), feline infectious peritonitis virus (FIPV), etc., which cause severe epidemiological problems in livestock and other domestic animals. Human coronaviruses are responsible for a significant number of common colds and diarrhoea, and have also been implicated in multiple sclerosis. Another member of the virus group, mouse hepatitis virus (MHV), is a frequent contaminant of laboratory mouse colonies.

Coronaviruses share several common

morphological and structural features.¹ They consist of an enveloped virus particle with characteristic petal-shaped spikes, which give an appearance of a crown, or corona, thus giving the name for the virus. The viral envelope contains two glycoproteins, one of which forms the spikes and the other the matrix proteins. The spikes interact with virus receptors on the cell surface, thus determining the target-cell specificity of the virus. Inside the viral envelope is a helical nucleocapsid which is composed of a nucleocapsid protein N and the RNA genome. The genome is a single piece of RNA of more than 18,000 nucleotides. The RNA is of positive sense, meaning that the viral genome can be directly translated into proteins.

Replication Pathway of Coronaviruses

Coronaviruses generally infect cells of the same animal species of origin in tissue culture. Immediately after the virus enters infected cells, the viral RNA is released from the virus particles by a process termed 'uncoating'. The viral RNA is used as messenger for an RNA-dependent RNA polymerase, which transcribes the genomic RNA into a full-length negative-stranded RNA.² This negative-stranded RNA species then serves as the template for the synthesis of seven virus-specific mRNAs. Each mRNA is used for the synthesis of either a viral structural or nonstructural protein. The nonstructural proteins are probably used to regulate the replication and transcription of virus. One of these nonstructural proteins is the RNA-dependent RNA polymerase which is needed to syn-

thesize more mRNAs as well as virion genomic RNA. The structural proteins, together with the genomic RNA, are used to assemble the virus particles. These particles, unlike most other enveloped viruses, bud into the endoplasmic reticulum, instead of directly into the plasma membrane.³ The mature virus particles travel through the Golgi complex and are eventually released into the extracellular space. The virus acquires the virus-specific glycoproteins, E1 and E2, during the budding and transport process.

Coronavirus mRNAs Have a Unique Nested-set Structure with a Common Leader RNA Sequence

Coronavirus mRNA is comprised of a genomic-sized RNA and six subgenomic mRNA species. All of these RNAs are associated with polysomes, thus representing functional mRNA species

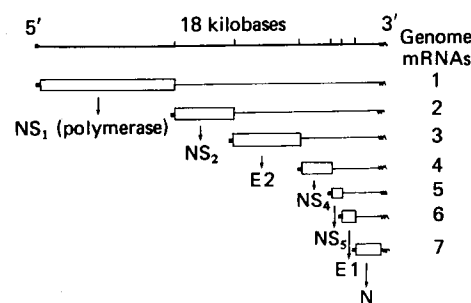


Fig. 1. mRNA structure of coronaviruses. The structure is based on that of mouse hepatitis virus. The open boxes represent the functional parts of mRNAs. The solid squares are the leader sequences and the wavy lines represent poly A tails. NS proteins represent nonstructural proteins which are not packaged into virus particles. E1, Matrix protein; E2, spike protein; N, nucleocapsid protein.