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ENCAPSIDATION AND TRANSDUCTION OF CELLULAR GENES BY RETROVIRUSES

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1. ABSTRACT

Retroviruses normally package their genomic RNA with high fidelity. However, the fidelity is apparently imperfect, since some cellular mRNA is present in standard retrovirus particles. Further, transcripts originating in the 5' LTR of the integrated provirus sometimes extend beyond the 3' end of the provirus, resulting in the production of chimeric RNAs containing both viral and cellular sequences. These RNAs can be exported to the cytoplasm and packaged into assembling virus particles. When such particles infect a new host cell, reverse transcriptase may copy the cellular sequences, as well as viral sequences, into DNA. In turn, recombinational events during reverse transcription can result in the incorporation of cellular sequences into retroviral genomes. If the cellular sequences encode proteins involved in the control of cell growth, then the high or inappropriate expression of these sequences as part of the retroviral genome may cause the malignant transformation of the infected cell. Viruses of this type, that transduce cellular transforming genes, are known as acute transforming viruses. They can only arise in animals infected with replication-competent retroviruses, and in general cannot produce progeny viruses without replication-competent "helper" viruses. Since they are produced by a complex, multi-step pathway, acute transforming viruses are only generated at very low frequencies.

2. INTRODUCTION

The transduction of cellular genes by retroviruses is a well-established phenomenon. Indeed, it played a crucial role in the development of our understanding of the origins of cancer. Thus, oncogenes were first identified as the transforming genes present in genomes of acute transforming retroviruses. It was subsequently learned that these transforming genes originate from cellular genes; their transforming capability arises as a result of mutations or other changes in the gene, and/or the inappropriate expression of the gene under the control of the viral promoter/enhancer machinery.

While the expression of the cellular gene as part of the transducing virus is very efficient, the generation of new transducing retrovirus genomes is a complex, multi-step process. It should be emphasized that the appearance of new transducing viruses is, in general, an extremely rare event. In the present review, we will discuss the aspects of retrovirus biology that enable them to give rise to transducing viruses and the possible mechanisms involved.

Retroviruses normally package their genome in the form of a dimer, consisting of two identical (+) strand RNA molecules, joined at or near their 5' ends by a limited number of base pairs. The viral genome has the structure of a cellular mRNA molecule, with a cap at its 5' end and a polyA tract at its 3' end. Indeed, RNA extracted from virions can be translated *in vitro* into the Gag and Gag-Pol proteins of the virus.

The Gag protein is the principal structural protein of a retrovirus particle. Expression of this protein is sufficient to form budding virus particles in mammalian cells, even in the absence of any other viral constituent (1).

Selection of the genomic RNA for packaging in the nascent particle is not understood in detail, but involves

recognition by the Gag protein of a cis-acting "packaging signal", frequently termed psi, in the 5' leader region of the RNA (reviewed in (2)). The recognition is evidently mediated by the nucleocapsid (NC) domain of the Gag protein, since mutations in this domain frequently prevent the specific packaging of the viral RNA (reviewed in (2)). However, encapsidation of psi+ RNA is completely unnecessary for efficient virus assembly (3, 4).

It has been recognized for many years that the preference for encapsidation of psi-containing RNAs is not absolute. Thus, as will be discussed below, at least some cellular mRNA species are present at low levels in normal, wild-type retroviral preparations. This encapsidation of cellular RNAs may be a prerequisite for the generation of transducing retroviruses.

We begin this review by discussing the packaging of cellular RNAs in retrovirus particles. We will then describe possible mechanisms of non-homologous recombination that might give rise to transducing retroviruses. Finally, we will briefly evaluate transduction as a safety concern in retrovirus-mediated gene transfer and gene therapy.

3. ENCAPSIDATION OF CELLULAR RNAS BY RETROVIRUSES

Retroviral particles contain not only the genomic RNA, but also cellular RNAs, such as 5S, 7S RNA, messenger RNA, even ribosomal RNAs, and especially significant amounts of tRNAs (for a review, see(2)).

3.1. Small cellular RNAs

Small cellular RNAs form a substantial fraction (roughly 20%) of the total RNA in a normal retrovirus particle. The small RNAs include the tRNA that is annealed to the viral RNA, where it will serve as primer for reverse transcription when the virus infects a new host cell (for a review, see (2)). The remaining small RNAs include 30-50 additional tRNA molecules, 5S rRNA, and the 7S RNA present in signal recognition particles (5). The functions and biological significance of these additional small RNAs in the virus are unknown. We have recently shown that RNA is a structural element in the retrovirus particle, serving as "scaffolding" to which Gag proteins bind in virus assembly (6). It is conceivable that the small RNAs contribute to the structure of the particle in this way.

3.2. High-molecular-weight RNAs

Both ribosomal and messenger RNAs from the host cell are found in retrovirus particles. The rRNA molecules may be present in the virion as components of intact ribosomal subunits. The level of rRNA is generally quite limited, and has been estimated to represent no more than one ribosome per virus particle (7).

The incorporation of cellular (or chimeric—see below) mRNA molecules into retrovirus particles is almost certainly a critical step in the formation of retroviruses capable of transducing cellular genes. Encapsidation of a cellular mRNA species was first described in 1974, when Ikawa *et al.* showed that murine leukemia virus (MuLV) particles produced by erythroid precursor cells contain small amounts of globin mRNA (8).

We have recently examined the RNA content of Moloney murine leukemia virus (MuLV) particles in some detail (6). Several of our findings are consistent with prior work on an exceptional avian cell line termed SE21Q1b (9). This cell line contains an avian sarcoma virus genome with a deletion in its packaging signal (10), and because of the high level of viral protein expression, packages large amounts of cellular mRNA (11). The present state of our knowledge concerning encapsidation of cellular mRNAs can be briefly summarized as follows.

1. Particles formed by wild-type Gag protein in cells lacking psi-containing RNA contain other mRNA molecules in place of the viral genome. Thus, all particles have similar amounts of total RNA. This RNA (whether viral or cellular) appears to be an integral part of the structure of the particle, since viral cores can be solubilized by exposure to RNase (6).

2. It is extremely difficult to detect these cellular mRNA molecules biochemically. This appears to be a result of their heterogeneity in size; a heterodisperse set of RNAs merely produces an indistinct "smear" in gel electrophoresis and is easily overlooked. In fact, the presence of these mRNAs was not obvious until an alphavirus vector-derived RNA was present in the cells from which the MuLV virus-like particles were produced. Under these conditions, the cellular mRNA population becomes almost monodisperse, because of the enormous amplification of the alphavirus-derived mRNA species. These discrete species are then easily detected in the MuLV virus-like particles (6).

3. Abundant cellular mRNA species can be readily identified in both wild-type and psi- particles using RT-PCR (6) or Northern blotting (12). We do not yet know whether packaging of cellular RNAs is "random"; in other words, the proportions of these species in virus particles may or may not be the same as their proportions in the cellular mRNA population. However, the two species tested (β-actin and glyceraldehyde-3-phosphate dehydrogenase) were in roughly the same proportions, relative to each other, in the cells, the wild-type particles, and the psi- particles. It was remarkable to find that the wild-type particles contain ~1/4-1/2 as much of these mRNAs as the psi- particles (6). If we assume that packaged mRNAs are a random sample of cellular mRNAs, then this result would indicate that wild-type particles contain a significant level of cellular mRNA; in fact, the high-molecular-weight RNA in a wild-type virus preparation could be a mixture of ~20% cellular mRNA and ~80% genomic RNA. Again, the heterogeneity of the cellular mRNA molecules would make them difficult to detect in direct biochemical analyses of RNA from virus particles.

Finally, this discussion of the encapsidation of cellular RNAs should also point out that the division of

mRNAs into "viral" and "cellular" is something of an oversimplification. The fact that retroviral genomes are integrated into cellular DNA introduces an inherent ambiguity into their status. Thus, endogenous proviruses (retroviral genomes inherited in chromosomal DNA) can be viewed either as cellular genes, since they are present in the germline, or as viral genomes, since their transcripts encode viral proteins and contain the cis-acting sequences essential for retroviral replication. Furthermore, the genomes of rats and mice also contain multiple copies of a retrovirus-like element called "VL30"; these give rise to RNAs that have the packaging signal and other cis-acting sequences required for retroviral replication, but do not encode proteins. As noted above, it is also not known whether the packaging of mRNAs lacking psi sequences is random. Perhaps some mRNAs, e.g., those with "psi-like" structures, are packaged far more efficiently than others. In other words, "viral" and "cellular" might represent the ends of a continuum rather than a pair of distinct, mutually exclusive alternatives for an mRNA molecule.

4. TRANSDUCTION OF CELLULAR GENES BY RETROVIRUSES

In order for a retrovirus to transduce a cellular gene efficiently, the gene needs to be incorporated into the retroviral genome by recombination. In principle, this recombination could occur at any of the following points in the retroviral life cycle: (1) transcription of nuclear DNA into RNA; (2) reverse transcription of RNA into DNA during infection of a new host cell; or (3) by recombination within the chromosomal DNA of the cell.

Our current understanding of the recombinational events that give rise to transducing viruses is based largely on analyses of the structures of acute transforming viruses. The cellular sequences (i.e., oncogenes) present in these viruses are always in the same transcriptional orientation as the viral genome itself. They also lack introns, suggesting that they arose by insertion of a stretch of mRNA, rather than genomic DNA of the host cell, into the viral genome. In a few acute transforming viruses, there is a poly(A) tract at the 3' end of the cellular sequences; this is further evidence that these sequences were acquired by recombination between cellular mRNAs and the viral genome.

It is important to note that, of the dozens of acute transforming viruses that have been characterized, all but Rous sarcoma virus (RSV) are replication-defective. That is, (except in RSV) the acquisition of the oncogene by the viral genome is accompanied by the loss of sequences encoding part or all of the Gag, Pol, and/or Env proteins necessary for the production of infectious retrovirus particles (13). These replication-defective viruses can only reproduce if the cell is co-infected with a "helper" virus, which supplies these viral proteins in trans. Of course, the transforming viruses are derived from replication-competent retroviruses; thus the helper is always present in the virus preparations along with the transforming virus. In fact, it can only be removed by complex virological procedures such as isolation of single-cell clones after infection of a culture at low multiplicity of infection.

4.1. Rous sarcoma virus: a unique example

Replication-competent avian retroviruses carrying the src oncogene, generically designated RSV, have been isolated repeatedly over the years. They contain src between the env coding region (the most 3' of the viral structural genes) and the 3' long terminal repeat (LTR), and the oncogene is expressed by splicing from the splice donor near the 5' end of the provirus to a splice acceptor just 5' of the src coding region. The different RSV isolates appear to have arisen by independent recombinational events between avian leukosis viruses and the src proto-oncogene (14). At present, we have absolutely no idea why acute transforming viruses containing the src oncogene are frequently replication-competent, while those with all other oncogenes are (to date) always replication-defective. In fact, src in RSV can be replaced with other oncogenes by recombinant DNA manipulations, resulting in a variety of other replication-competent acute transforming viruses (for example, (15)). Thus, viruses of the latter type can be created in the laboratory, but have not, to our knowledge, arisen *in vivo.*

4.2. Mechanisms of cellular gene transduction by retroviruses

It seems likely that many acute transforming viruses arose by the following pathway, outlined schematically in Figure 1. The length and complexity of this sequence of events make it clear why acute transforming viruses arise so infrequently, even in animals with persistent high-level viremia.

1. A replication-competent retrovirus integrates into chromosomal DNA on the 5' side of the cellular gene that will be incorporated into the acute transforming virus (this gene is termed a proto-oncogene ("c-onc") in its normal form in cellular DNA, and an oncogene ("v-onc") as part of the transforming virus) (Figure 1, a).

2. A chimeric RNA molecule, containing both viral and proto-oncogene sequences, is formed by transcription from the promoter in the 5' LTR of the provirus (Figure 1, b). The two sequences may be joined by "readthrough" transcription past the normal transcriptional termination signal in the 3' LTR into the proto-oncogene, or by deletion of the 3' end of the provirus (see example in Figure 2). Joining of the cellular and viral sequences in this chimeric RNA is the first of the recombination events in the pathway.

3. The chimeric RNA molecule is packaged into a viral particle together with a full-length molecule of genomic RNA (Figure 1, c).

4. The particle infects a new host cell (Figure 1, d). During reverse transcription, one or more additional recombinations take place Figure 1, e), resulting in the placement of the oncogene within the new, recombinant viral genome (i.e., between the LTRs, in a molecule

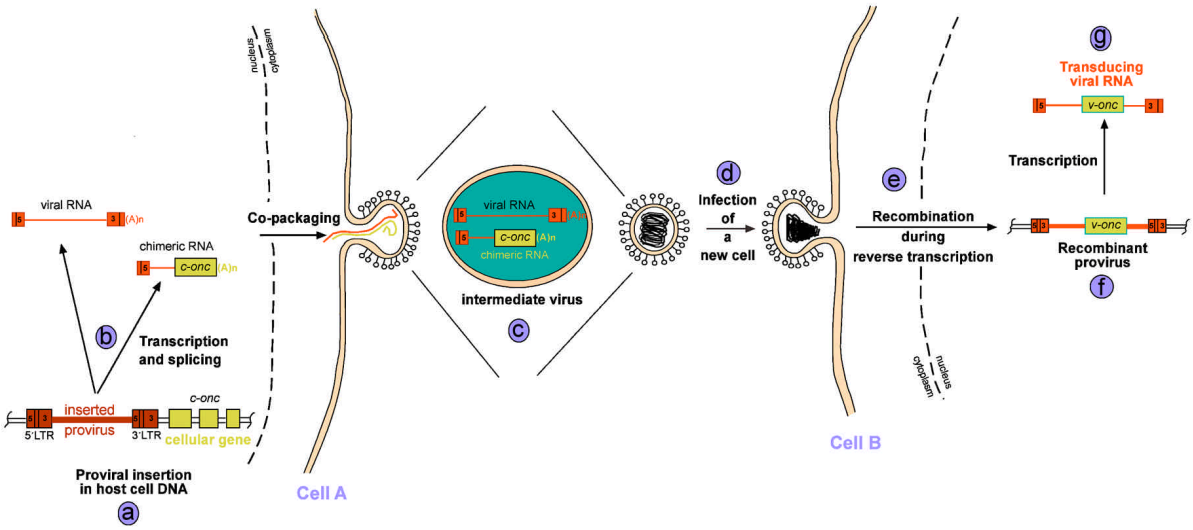

Figure 1: Transduction of cellular genes by retrovirus. (**a**) Insertion of the provirus in the host cellular DNA upstream from the transducible cellular gene (cellular oncogene, "*c-onc*"). (**b**) Retroviral transcription usually leads to the formation of the viral RNA, but sometimes to a chimeric RNA created by readthrough of the viral polyadenylation signal "(A)n", following by splicing (for an alternative, see Figure 2). (**c**) The viral genomic RNA (helper virus) and the readthrough transcript (chimeric RNA) are co-packaged into an intermediate virion. (**d**) The intermediate virus infects a new cell. (**e**) During the synthesis of the proviral DNA by the reverse transcriptase, non homologous recombination occurs: (**f**) the resulting recombinant provirus is inserted into the chromosomal DNA of cell B. This provirus carries sequences from the cellular gene of cell A, now called the viral oncogene ("*v-onc*"). (**g**) The last step involves the transcription of this DNA unit into a viral RNA able to transduce the viral oncogene from one cell to another. LTR: long terminal repeat.

containing all the cis-acting sequences required for replication) (Figure 1, f and g).

4.2.1. Readthrough transcription

Individual steps in this pathway have been studied in some detail in model systems in cell culture. Thus, "readthrough" transcription in avian proviruses was systematically analyzed by Herman and Coffin (16) and by Swain and Coffin(17). They found that the transcriptional termination sites at the 3' end of the provirus are rather inefficient, so that transcripts extending beyond them are easily detectable in both the cell and the virus. (On the other hand, almost no transcripts initiating in the 3' LTR were detected.) They also found that introducing a point mutation into the AAUAAA polyadenylation signal increased the frequency of readthrough transcription significantly.

The chimeric transcript may be longer than the genome of the parental, replication-competent retrovirus. Herman and Coffin(16) observed that RNAs considerably longer than the genome can be packaged with moderate efficiency. These findings were recently extended by Shin *et al.* (18), who detected encapsidation of RNAs more than twice the length of the wild-type genome into MuLV particles.

One particularly well-characterized example is that of transduction of erbB by avian leukosis viruses (ALV). As far as is known, the site of integration of the provirus is completely random. At a low frequency, therefore, ALV can be inserted upstream from the first exon of the cellular oncogene c-erbB in infected chickens (19). This insertion results in c-erbB activation by readthrough transcription and the generation of a chimeric RNA in which viral sequences are fused to truncated c-erbB mRNA (20). This configuration results in relatively efficient generation of erbB-transducing viruses (21).

4.2.2. Non-homologous recombination

The transduction process of cellular messenger RNA by retroviruses involves recombination steps during reverse transcription (Figure 1, e). This process requires co-packaging of the chimeric RNA, or a cellular RNA devoid of viral sequences, together with a helper virus genome (Figure 2). As noted above, retroviruses normally package a dimeric RNA, consisting of two identical copies of the genome (a homodimer). Genetic studies show that retroviral RNAs will only recombine if they have been packaged in the same virion (22), and thus, it has frequently been assumed that the viral and the chimeric RNA are initially linked in a heterodimer. However, there is no empirical evidence for or against this hypothesis, and heterodimers have never been observed to date.

During retroviral replication, reverse transcriptase-mediated recombination normally occurs between the two retroviral genomes and is partly responsible for the high genetic variability of retroviruses. Such recombination is usually homologous, but non-homologous recombination can also occur and lead to the production of provirus with substitutions, deletions, or

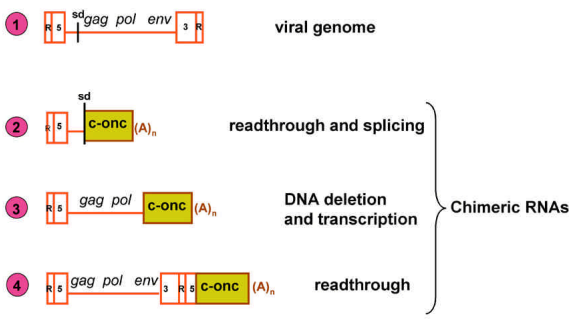

Figure 2. Transcripts that can lead to the transduction of cellular genes ("*c-onc*"). (**1**) Example of a viral genome that can serve as a helper during transduction. Chimeric RNAs are created by (**2**) readthrough transcription followed by fusion during splicing, or (**3**) DNA deletion, or (**4**) simple readthrough of the viral polyadenylation signal located in the 3'LTR. The chimeric RNA (2, 3 or 4) will be co-packaged into the virion with the helper viral genome (1) and will be transmitted to another cell for transduction (as shown in Figure.1).

insertions. This rate is 0.1 to 1% of the rate of homologous recombination (23). This event can occur at different steps of reverse transcription, during both minus and plus strand DNA synthesis (22).

Since non-homologous recombination can occur during the retroviral life cycle, recombinational events can generate transducing viruses even in the absence of a chimeric RNA. That is, recombination can occur between a viral RNA and a co-packaged cellular mRNA despite the absence of any regions of homology between the two RNAs. This was demonstrated in experiments on the exceptional quail cell line, SE21Q1b. Since virions produced by SE21Q1b cells contain cellular mRNAs (24), Linial and coworkers (25) tested the possibility that these virions could transduce a non-viral trait to new host cells (Figure 3). They transfected SE21Q1b cells with a plasmid encoding G418-resistance. This plasmid contained no retroviral sequences. They then found that virus from the transfected cells could transmit the gene for G418-resistance to new quail cells. However, the gene was expressed at very low frequency in the new host cells, presumably because it usually failed to integrate 3' to a constitutive promoter. In subsequent studies, Hajjar and Linial (26) showed that virus transducing the G418-resistance phenotype was reproducibly recovered from the transfected SE21Q1b cells only if the cells were also infected with a wild-type avian leukosis virus (ALV). Analysis of the transducing viral genomes showed that they were recombinants in which the neo gene was flanked by all of the cis-acting signals required for retroviral replication, with transcription of this gene driven by the 5' LTR. Formation of these recombinants cannot depend upon the chance integration of the provirus to the 5' side of the neo gene, since it occurred in a single replication cycle and in each of a number of cell clones. Thus, they arise by direct recombination between the two parental RNAs, with no requirement for a chimeric precursor RNA, despite the lack of homologous sequences in the two RNAs. It was also shown that this process, named "retrofection", required extracellular reinfection and apparently cannot occur intracellularly (27). Somewhat analogous studies in a MuLV-based system were performed by Stuhlmann *et al* (28).

Transduction of a nonviral gene by particles of spleen necrosis virus (a retrovirus related to MuLV) has also been analyzed in detail (29, 30). The transduced genes lacked poly(A) at their 3' ends, were truncated at both their 5' and 3' ends, and were not flanked in the chromosome by direct repeats. In all of these respects, they differed from natural processed pseudogenes; thus this does not seem to be the mechanism by which the latter are formed, although they must arise by reverse transcription of cellular mRNA molecules.

The non-homologous recombination described here almost certainly occurs during the synthesis of DNA in the newly infected cell, using the encapsidated RNAs as a template. What is the mechanism by which DNA synthesis on a cellular mRNA template is initiated? It has been known for many years that reverse transcription of a retroviral genome is initiated from a specific cellular tRNA that is annealed to the viral RNA. The nature and stringency of the requirement for the "correct" tRNA species has been extensively investigated. In general, retroviruses can be modified to use other tRNAs simply by changing the bases in the genome that interact with the tRNA (31-33). Taylor and Cywinski (34) investigated the nature of the primer used for initiation of DNA synthesis on the cellular mRNA molecules present in the virions from SE21Q1b cells. Interestingly, these DNA copies are evidently also initiated from tRNA molecules, despite the fact that the cellular mRNAs almost certainly lack stretches of perfect complementarity to these tRNAs. Only a small fraction of the tRNA primers used in DNA synthesis from SE21Q1b-derived virions are $tRNA^{Trp}$, whereas essentially all DNA synthesis from wild-type avian retroviruses is initiated with this one tRNA species.

Interestingly, the reverse transcription of 7S L RNA was also observed in cells newly infected with virions from SE21Q1b cells (35). The initiation of reverse transcription, as with viral RNA, occurs on a tRNA-like primer complementary to an internal sequence in the 7S RNA sequence: a 135 base strong-stop DNA product is produced in these particles, and recombinant retroviruses containing 7S L sequence were obtained. While the role, if any, of 7S RNA in the retroviral life cycle remains unknown, it has been proposed that retrovirus-mediated reverse transcription of 7S L RNA and its subsequent integration in the host DNA may be a step in the generation of mammalian Alu-like elements (35).

4.2.3. DNA recombination

In addition to RNA recombination and readthrough transcription, another mechanism for transduction of cellular genes by retroviruses cannot be excluded at present: recombination at the DNA level. However, this would probably occur at a much lower rate than RNA recombination. Using defective, truncated

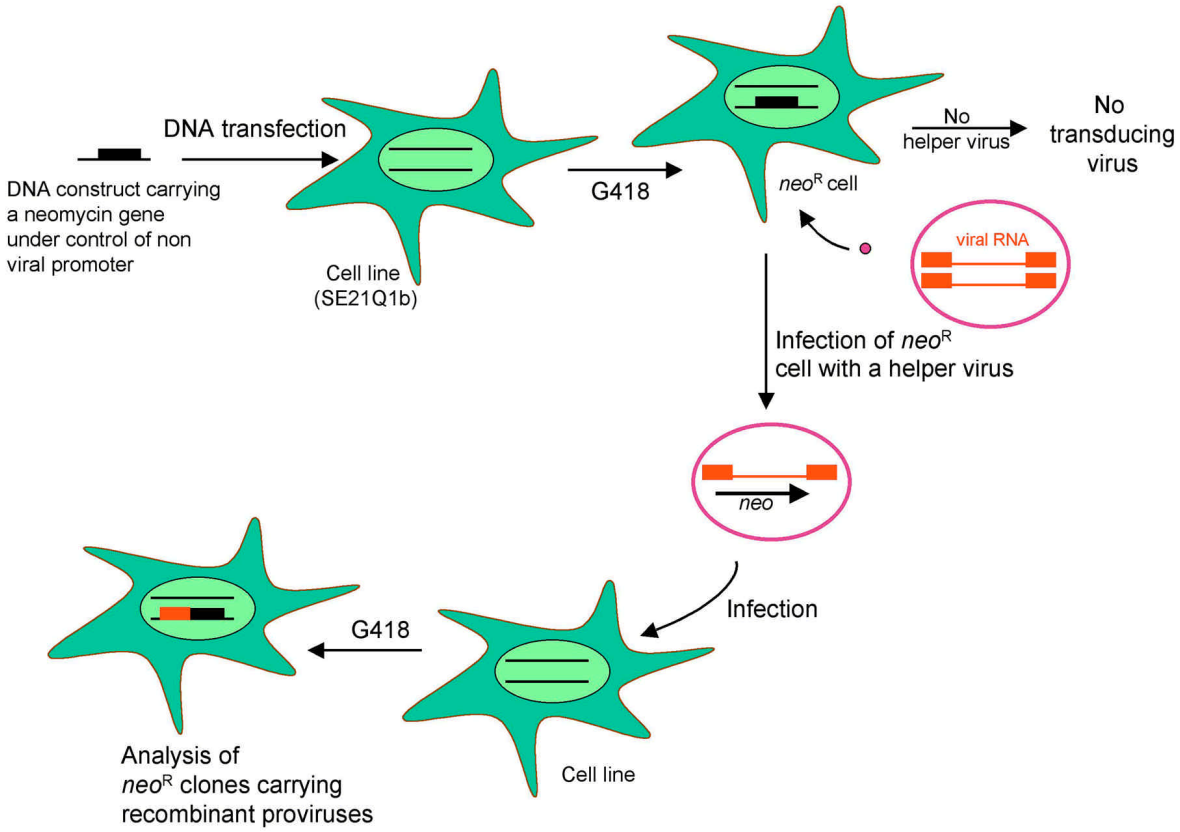

Figure 3. Schematic procedure to study the formation of transducing virus: principle of retrofection. (adapted from Linial and coworkers).

Harvey sarcoma virus proviruses as models for transduction intermediates of the ras oncogene, Goodrich & Duesberg (36) suggested that the regeneration of infectious Harvey viruses involved rare, non-homologous recombination between proviral and cellular DNA.

4.3. Participation of endogenous retroviral and viral-like sequences in transduction

As indicated above, mammalian cells contain retroviral genomes ("endogenous viruses") in their chromosomes; they also contain retrovirus-like elements such as VL30. The RNA genomes of endogenous viruses and retrovirus-like elements are efficiently encapsidated into retrovirus particles, and it seems plausible that they might contribute in some way to the transduction of cellular genes. Indeed, in some systems, there is direct evidence for participation of retrovirus-like elements in transduction. Thus, Harvey sarcoma virus (HaSV), an acute transforming virus carrying the ras^H oncogene, originally arose in a rat infected with Moloney murine leukemia virus (Mo-MuLV). However, the HaSV genome is actually a "double recombinant": the extreme termini of the genome are derived from MoMuLV, as expected, but internal to these Mo-MuLV sequences are VL30-derived sequences, on both the 5' and 3' sides of the oncogene (37, 38).

5. A COMMENT ON SAFETY

Retroviruses have been widely considered as possible gene therapy vectors. One of the obstacles to their use up to now has been concern about the possibility that acute transforming viruses will be generated in the patient and will give rise to malignant disease. However, it should be clear from the foregoing that such viruses are only formed by a complex, multi-step pathway. Although the number of existing acute transforming viruses carrying an oncogene may seem large, it is actually minuscule compared to the numbers of retrovirus-infected mice, rats, and other experimental animals that have been observed in the laboratory over the last several decades. Further, as far as is known, these viruses can only originate following infection with a conventional, replication-competent parental retrovirus, while the retroviral vectors that might be used in gene therapy would presumably be replication-defective. In our opinion, the risk posed by new acute transforming retroviruses thus seems extremely small.

Another possible hazard in gene therapy with retroviruses is the activation of proto-oncogenes by integration of the vector at nearby sites in the cellular DNA. This is a real possibility, but it should be

remembered that the number of vector particles introduced into the patient is very small compared with the number of potential integration sites in the cellular genome. Thus, as long as the vector cannot replicate in the patient, the probability of insertion next to a proto-oncogene is small.

A far greater source of concern is the possible presence of replication-competent retroviruses (RCR) in retroviral vector preparations. RCR can arise by recombination between the vector genome and sequences in the cells used to produce the vectors; such viruses can be difficult to detect. RCR cause malignancies with high efficiency in immunosuppressed hosts, principally by integrating near proto-oncogenes and activating their expression (13). Indeed, pathogenicity of RCR accompanying "therapeutic" retrovirus particles has been documented in monkeys (39, 40) as well as in mice and chickens (13). Safety considerations for the use of retroviral vectors in human gene therapy are considered in detail elsewhere in this volume.

6. CONCLUDING REMARKS

Despite the decades of intensive investigation of transducing retroviruses, we still do not fully understand the "rules" governing their formation. It is striking that they have only been described as originating from "simple" retroviruses (i.e., those expressing only Gag, Pol, and Env proteins), and not from lentiviruses or from complex oncoretroviruses such as bovine leukemia virus. It is also remarkable that all acute transforming viruses are replication-defective, with the unique exception of multiple isolates of Rous Sarcoma Virus. As noted above, both transducing lentiviruses (for a review, see (41)) and replication-competent viruses with oncogenes other than src have been constructed in the laboratory by recombinant DNA techniques. Further work in experimental animals may be required in order to determine the nature of the barriers to the formation of such viruses *in vivo*.

It is also striking to note that acute transforming viruses are never observed in nature, despite the existence of natural populations of mice, cats, and chickens with high levels of RCR. Since acute transforming viruses are known to arise in viremic animals in the laboratory, their absence in natural populations suggests that they are strongly selected against in these populations.

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