Key words: *cloning vectors/animal viruses/gene expression*

REVIEW ARTICLE

Cloning Vectors Derived from Animal Viruses

By PETER W. J. RIGBY

Cancer Research Campaign Eukaryotic Molecular Genetics Research Group, Department of Biochemistry, Imperial College of Science and Technology, London SW7 2AZ, U.K.

INTRODUCTION

The use of autonomous replicons derived from plasmids and bacteriophages in the construction of cloning vectors for use in prokaryotic cells has been essential to the revolution in biological knowledge which has occurred since the advent of recombinant DNA technology. A large variety of such prokaryotic vectors, many of them tailored to particular experimental purposes, is now available. When applied in concert these vectors enable the isolation of any gene, given a suitable screening or selection procedure; they facilitate the determination of its nucleotide sequence and they can be employed to force the expression of some genes in foreign environments. Such studies have revealed many surprising features of the organization of eukaryotic genes and it has been possible, by comparing the nucleotide sequences of putative control regions, to identify elements likely to be involved in the mechanism or regulation of gene expression; for example, the sequence TATA(A/T)A(T/A), or a variant thereof, is found approximately 30 nucleotides upstream of the initiation site of most RNA polymerase II transcription units (Goldberg, 1978; Corden *et al.*, 1980) and has therefore been implicated as a part of eukaryotic promoters. It has similarly been possible to identify consensus sequences associated with the polyadenylation of mRNA and with RNA splicing. However, mere inspection of nucleotide sequences can not prove a relationship between structure and function and it is clear that comparative sequence analysis will never reveal all aspects of gene structure and function. Functional assays for gene expression which permit genetic analysis are absolutely required if our ever-increasing knowledge of gene structure is to be correlated with biological function. If the 'TATA' sequence is crucial to the correct initiation of transcription by RNA polymerase II, then mutations in this sequence must disturb initiation and it is only by exploring the effects of a large number of such mutations that we can learn the precise requirements of such putative control sequences. There now exist many techniques for the precise mutation of cloned genes *in vitro* so the generation of the requisite mutants is not a limiting factor. Such mutants must, however, be analysed in a system which is biologically significant; one must be able to return the cloned gene and its mutant derivatives to an environment which mimics as closely as possible that in which the gene normally functions.

In vitro systems for gene expression are clearly very convenient technically and are absolutely required for the final characterization of a system in which accurate expression and correct regulation are reconstituted from purified components. However, it is not at all clear that such *in vitro* systems are ideal for initial studies. For example, there now exist *in vitro* systems for the transcription of cloned genes by RNA polymerase II, but such systems are restricted to initiation; correct termination and/or polyadenylation have not been documented and there are no convincing data for *in vitro* splicing. It is also likely that such systems, in which the template for transcription is naked DNA, will not be useful for the study of regulatory phenomena which depend on chromatin structure. What is required is the development of procedures by which cloned genes can be reintroduced into cultured eukaryotic cells and these procedures should ideally be general, so that each gene can be transferred into the cell type is which it is normally expressed. Appropriate vector systems must thus be developed and in the absence of well-defined plasmid DNAs in eukaryotic cells animal viruses have provided the obvious basis for such vectors. The need for, and utility of, such *in vivo* assays for gene expression is exemplified by

0022-1317/83/0000-5459 $02.00 © 1983 SGM

the fact that particular mutations in the promoter of the early transcription unit of simian virus 40 (SV40) have different effects when assayed *in vivo* and *in vitro* (Benoist & Chambon, 1981; Mathis & Chambon, 1981).

The general principles governing the manipulation of eukaryotic viral genomes during vector construction are closely analogous to those which have been employed during the development of the extensive series of bacteriophage λ vectors currently available (Williams & Blattner, 1980; Brammar, 1982). The vector genome must be easily manipulated, it must contain convenient sites for the restriction enzymes used in cloning and the location of these sites with respect to vector control sequences must be well understood. This last point is of crucial importance because the primary requirement for any eukaryotic cloning vector is that efficient expression of the inserted gene be guaranteed. As with bacteriophage vectors, many animal virus vectors are subject to rigid packaging constraints. This is a particular problem when the vector is to be used to analyse the expression of eukaryotic genes because many chromosomal genes are very large, for example the mouse dihydrofolate reductase (DHFR) gene is longer than 42 kb (Nunberg *et al.*, 1980). Such genes can therefore only be carried in viruses, for example herpes simplex virus (HSV), capable of packaging a large genome and the large size of the viral genome and its complex pattern of gene expression introduce technical problems in its use as a vector. In order to avoid such packaging constraints, it is highly desirable to develop vectors which replicate episomally and thus do not need to pass through an infectious virion. The restricted host range of most animal viruses also presents a problem; the ideal vector would be capable of delivering the cloned gene into many specialized cell types derived from various species. Considerable emphasis has therefore been placed upon techniques for introducing unpackaged DNA into cultured eukaryotic cells.

The most commonly employed procedure is to co-precipitate the DNA with calcium phosphate (Graham & van der Eb, 1973), a technique generally referred to as transfection, even in the many cases in which it does not lead to an infection. There are no *in vitro* packaging systems for animal viruses, so even in those cases in which the recombinants are to be propagated as infectious virions the initial infection must be done by DNA transfection. When virions are produced, the efficiency of transfection is not a limiting factor as the recombinant virions can subsequently be used to guarantee efficient infection. However, if the recombinant can not pass through the viral life cycle and is to be replicated episomally or as a chromosomally integrated segment, the efficiency of transfection can be limiting. Moreover, recent years have seen the extensive use of so-called transient expression assays in which the recombinant DNA is transfected into animal cells and the expression of the exogenous gene(s) is assayed a few days later. In such systems, DNA replication is not required and again efficient transfection is vital. Subtle technical changes in the original co-precipitation procedure can considerably increase the percentage of cells which take up DNA (Chu & Sharp, 1981) but many cells are extremely refractory to transfection. The most promising procedure for circumventing this problem is protoplast fusion (Schaffner, 1980) in which protoplasts derived from *Escherichia coli* carrying the recombinant DNA molecule are fused with cultured eukaryotic cells using polyethylene glycol. Rassoulzadegan *et al.* (1982) have refined this technique so that all of the cells in the culture take up and express the exogenous DNA.

Although transient expression assays are very convenient, in many cases one wishes to construct a cell line which stably expresses the foreign gene. Even with the availability of highly efficient gene transfer techniques this requires selective systems, analogous to those used in prokaryotic cloning, which ensure that only those cells which have taken up and are expressing the foreign DNA survive. The development of such selective systems will be a particular focus of this review. An inherent disadvantage of most virus vectors is that they lead to the death of the infected host cell. Virus vectors which pass through the lytic cycle can be advantageous when the object is high-level expression of a gene product, but in many cases it is preferable to construct a cell line which stably expresses the foreign gene, thus obviating the need for repeated viral infections and I shall discuss several procedures for achieving this objective. It is not possible in this short review to discuss in detail all of the animal virus vector systems which have been described. I shall concentrate on defining the basic principles which govern the use of animal

virus genomes as vectors and on reviewing some recent results which illustrate what appear to be the most profitable routes for future research. The use of SV40 as a cloning vector has been considered in detail by Elder *et al.* (1981) and I have recently discussed at much greater length many of the points covered in this review (Rigby, 1982). There is also an up-to-date monograph which considers all aspects of the design and use of eukaryotic virus vectors (Gluzman, 1982).

Vectors for the propagation of recombinant genomes as virions

SV40 was the first eukaryotic DNA virus for which a complete nucleotide sequence and a detailed understanding of its transcription were available (Tooze, 1981) so it is not surprising that this virus formed the basis of the first eukaryotic cloning vehicles. The genome of SV40 contains very little non-essential DNA so it is necessary to insert the foreign gene in place of essential viral genes and to propagate the recombinant genome in the presence of a helper virus. The original procedure for doing this was to perform a mixed infection between a recombinant in which viral late gene expression is defective and a helper virus carrying a temperature-sensitive mutation in an early gene. Following infection at the non-permissive temperature, growth can occur only by complementation between the helper virus, which provides late functions, and the recombinant, which provides early functions. Early work using this approach (Goff & Berg, 1976, 1979; Hamer *et al.*, 1977) showed that the SV40 replicon could be used to propagate foreign DNA in cultured eukaryotic cells but expression of the inserted DNA was not observed. This lack of expression was readily explained by the knowledge that SV40 late mRNAs are spliced; in the first generation of late replacement vectors the splicing signals of the late transcription unit were deleted. Once the location of the splice junctions had been established, it was relatively straightforward to design vectors which ensured that transcription from the SV40 late promoter yielded hybrid mRNAs which were properly processed and expression of the inserted gene was then observed (Mulligan *et al.*, 1979). Such late replacement vectors have been used to transduce into cultured eukaryotic cells a variety of genes and cDNAs, for example those coding for rabbit β-globin (Mulligan *et al.*, 1979), mouse α- and β-globins (Hamer & Leder, 1979*a*, *b*, *c*; Hamer *et al.*, 1979, 1980), rat preproinsulin (Gruss & Khoury, 1981, Gruss *et al.*, 1981*b*), the surface antigen of human hepatitis B virus (Moriarty *et al.*, 1981) and the haemagglutinin of influenza virus (Gething & Sambrook, 1981; Sveda & Lai, 1981; Hartman *et al.*, 1982). In general, foreign genes have been inserted into the late region of SV40 because higher levels of expression are obtained from the late promoter than from its early counterpart. However, an alternative strategy for the provision of helper function has recently given an added dimension to the use of early replacement vectors. CV-1 cells, a line of African green monkey kidney cells, are permissive hosts for SV40 and so to obtain transformation it is necessary to inactivate viral DNA replication by mutating either the gene for large T-antigen (the viral protein involved in the initiation of viral DNA replication) or the viral origin. Gluzman (1981) introduced into the origin a deletion which does not affect the early promoter and then used this cloned early transcription unit to transform CV-1 cells. The resultant COS cells synthesize both functional large T-antigen and the permissivity factors required for efficient viral DNA replication yet the origin deletion means that the resident viral genome can be neither replicated nor rescued. Recombinant viruses in which the foreign gene has been inserted into the early transcription unit will thus replicate in COS cells under the influence of the large T-antigen synthesized from the integrated viral sequences (Gething & Sambrook, 1981). This system has the considerable advantage that a pure recombinant virus stock can be produced, an advantage that for many purposes outweighs the lower level of expression obtained from the early promoter.

However, all work using SV40 virions to propagate recombinant DNA molecules is severely constrained by the facts that the viral genome is small, 5·24 kb, and that the packaging limits are strict. Such systems can not, therefore, be used for the analysis of most eukaryotic genes and much recent work has therefore been concentrated on the development of the episomal and integrating SV40 vectors discussed below.

Human adenoviruses have been intensively studied in recent years because of the inherent interest of their molecular biology and because they are tumour viruses. The large amount of

background information that has been acquired (Tooze, 1981) is now being applied to the development of adenovirus vectors. The manipulation of the adenovirus genome is intrinsically more difficult than the manipulation of SV40 DNA because the former is considerably larger (approximately 35 kb), it is linear and it carries a covalently attached terminal protein. The larger size of the adenovirus genome means that it generally contains multiple sites for the restriction endonucleases used in cloning and thus, in order to manipulate the genome effectively, it has been necessary to eliminate certain restriction sites. This has been achieved by the adaptation to adenoviruses of the techniques so effectively utilized for the construction, *in vitro*, of deletion mutants of SV40 and by the exploitation of the observation that adenovirus stocks contain variants, lacking restriction sites. These variant adenovirus genomes can be isolated following successive cycles of restriction and re-ligation (Jones & Shenk, 1978, 1979). Another major development in the use of adenoviruses as vectors is due to the work of Stow (1981) who showed that cloned viral DNA fragments can be reintroduced into viral genomes. It is possible to clone a DNA fragment from the left terminus of adenovirus 2, mutate it *in vitro* and then reincorporate it into a virus by ligating it to a large DNA fragment, containing the rest of the viral genome, derived from the adenovirus 5 deletion mutant *dl*309 (Jones & Shenk, 1979) which contains a single *Xba*I site. Adenoviruses have a mechanism by which one of the inverted terminal repeats can be restored so long as the other is intact and thus in such constructions it is not necessary to separate the manipulated terminal fragment from the plasmid vector (Stow, 1981, 1982*a*). In this way it should be possible to construct, by manipulation in *E. coli*, hybrid transcription units in which foreign genes are placed under the control of the adenovirus El early transcription unit, which occupies the left-terminal 12% of the genome, and then to reincorporate them into intact adenoviruses; similar procedures could be developed for other regions of the adenovirus genome. Insertion of foreign DNA will inactivate the El region but helper function can be provided by the integrated adenovirus sequences carried in the chromosomes of strain 293 cells (Graham *et al.*, 1977). These are human embryonic kidney cells transformed by sheared adenovirus 5 DNA and which express El functions. The application of presently available gene transfer techniques will allow the construction of other cell lines in which the adenovirus functions required to help recombinant viruses are provided from chromosomally integrated sequences (Grodzicker & Klessig, 1980).

Adenoviruses have already been used as vectors in order to obtain the expression of large amounts of SV40 large T-antigen (Thummel *et al.*, 1981; Solnick, 1981). In these experiments less precise vector design can be employed because the desired recombinants can be selected genetically. The infection of monkey cells by human adenoviruses is abortive but the block can be overcome if the cells are co-infected with SV40 (Tooze, 1981); adenovirus–SV40 hybrid viruses which express at least the C-terminal portion of SV40 large T-antigen can thus grow in monkey cells. Expression of the large T-antigen gene from the adenovirus major late promoter results in the synthesis of large amounts of the protein thus facilitating its purification and analyses of its biochemical functions (Myers *et al.*, 1981).

Retroviruses have single-stranded RNA genomes and would thus at first sight appear to be unpromising candidates as vectors but the peculiarities of the retrovirus life cycle mean that these viruses provide perhaps the most promising vector system of all. During the process of reverse transcription, which generates double-stranded proviral DNA, sequences from the termini of viral RNA are duplicated to generate the long terminal repeats (LTRs). These LTRs contain both the promoter and the polyadenylation signal for the transcription of viral mRNAs and of genomic RNA and also determine the specificity of proviral DNA integration (Weiss *et al.*, 1982). Although retroviruses can integrate at many sites within the cellular genome, integrative recombination always occurs at particular sites at the ends of the LTRs and thus one can be assured that sequences appropriately inserted between the two LTRs will be integrated intact. This contrasts sharply with the integration of papovavirus or adenovirus DNA, during which extensive rearrangements of the integrated viral sequences are commonplace. A further great advantage of retroviruses is that they are natural transducing viruses. The strongly transforming retroviruses which have been so intensively studied in recent years were generated by the incorporation of cellular genes, or of mRNA transcripts thereof, into non-transforming,

replication-competent retroviruses. Analyses of the structure and expression of the genomes of transforming retroviruses (Weiss *et al.*, 1982) have thus served to define in considerable detail the rules governing the expression of foreign genes incorporated into retrovirus genomes. Additional advantages of retroviruses are that they have relatively wide host ranges, which can be extended by pseudotyping, and that retrovirus infection does not lead to immediate cell death. Cells transformed by retroviruses continue to grow and divide while producing large amounts of infectious virions.

Several groups have now described model experiments in which foreign genes have been incorporated into retrovirus genomes by *in vitro* manipulations. The favourite foreign gene has been the thymidine kinase (tk) gene of HSV, because expression of this gene can be selected for after infection or transfection of tk^{-} cells. Wei *et al.* (1981), Shimotohno & Temin (1981) and Tabin *et al.* (1982) have used Harvey murine sarcoma virus, spleen necrosis virus and Moloney murine leukaemia virus, respectively, as vectors to construct recombinant genomes capable of transducing the HSV tk gene. The recombinant genomes thus produced are, like most strongly transforming viruses, replication-defective because the exogenous gene is inserted in place of one or more of the viral genes required for replication. Such recombinant genomes can be introduced into cells by transfection and the recombinant virus subsequently rescued from the integrated state by superinfection with a replication-competent helper retrovirus.

The HSV tk gene does not contain introns and its expression therefore requires no additional processing steps. Because of the established relationship between the oncogenes carried in transforming retroviruses and the cellular proto-oncogenes from which they are derived (Weiss *et al.*, 1982) it seemed likely that the incorporation of an intron-containing gene into a retrovirus vector should lead to the elimination of the introns. Shimotohno & Temin (1982) have shown that this is indeed the case. They cloned the mouse α-globin gene, which contains two introns, into their spleen necrosis virus vector and demonstrated that the two introns are precisely excised. Thus the use of retrovirus vectors will not be confined to cDNA or to intronless genes.

Thus far, retroviruses have only been used as vectors for genes which had previously been isolated by cloning in *E. coli*. However, the fact that retroviruses express foreign genes so efficiently means that they have considerable attraction for the development of systems in which the foreign gene is recognised by virtue of its expression in eukaryotic cells. One can confidently expect the construction of retrovirus vectors suitable for use in primary cDNA cloning experiments.

Recent work has shown that herpes simplex and vaccinia viruses may also form the basis of useful vector systems. In both of these cases the viral genome is extremely large and our understanding of its expression is limited but, as noted above, vectors derived from such viruses may be necessary to analyse the expression of very large eukaryotic genes. Studies of the defective genomes generated during the repeated passage of HSV stocks have allowed Spaete & Frenkel (1982) to define a cloned segment of HSV DNA which will both replicate in cells co-infected with a helper virus, which provides viral DNA replication functions, and become packaged into viral particles. Stow (1982*b*) has cloned a small fragment of HSV type 1 DNA which contains a viral origin of DNA replication but not the packaging signals and shown that plasmids carrying this fragment will replicate in HSV-infected cells. Further definition of the precise sequence requirements for HSV DNA replication and packaging, and the incorporation into such recombinants of well-characterized promoters, should lead to the development of effective vector systems. Panicali & Paoletti (1982) have shown that it is possible to construct recombinant poxvirus genomes although in this case, because poxvirus DNA is non-infectious, it is necessary to perform the final stage of construction by *in vivo* recombination. They inserted the HSV tk gene into a cloned segment of vaccinia DNA and demonstrated that if the resulting plasmid is transfected into vaccinia virus-infected cells, infectious virions carrying the HSV tk gene are produced and the tk gene is expressed in cells infected by such recombinant virions.

Virus-based vectors which replicate episomally

The development of vectors which replicate episomally in eukaryotic cells would be of great advantage because such vectors are not subject to packaging constraints nor need they lead to

the production of infectious virions and thus it should be possible to use them to construct stable cell lines which express the gene of interest.

Papillomaviruses are quite distinct from the related papovaviruses in that papillomavirus-transformed cells do not contain integrated viral DNA (Law *et al.*, 1981; Moar *et al.*, 1981 *a, b*). Rather, they contain between 50 and 300 copies of unintegrated, circular viral DNA although some proportion of these viral genomes exists as concatamers and/or catenates. The virus which has received most attention as a vector is bovine papillomavirus type 1 (BPV-1), which can efficiently induce the morphological transformation of the C127 line of mouse fibroblasts. Only 69% of the viral genome is required for transformation (Lowy *et al.*, 1980) and Sarver *et al.*, (1981 *a, b*) showed that this fragment could be used as a vector to introduce the rat preproinsulin gene into C127 cells. The transformed cells contained the recombinant genome as an episome, synthesized preproinsulin mRNA which had authentic 5′ termini and secreted proinsulin into the culture medium. Similar constructions have been performed by Zinn *et al.* (1982) using the human β-interferon gene; the transformed cells synthesize and secrete human β-interferon and, interestingly, transcription of the interferon gene can be induced by poly(I)·poly(C). In all such work, the BPV-1 vector fragment is propagated as a plasmid clone in *E. coli* and the plasmid vector sequences must be removed prior to the transformation of C127 cells because they inhibit this process. It would clearly be advantageous to have a shuttle vector which can replicate efficiently in both eukaryotic and prokayotic cells. DiMaio *et al.* (1982) have described just such a vector in which the transforming fragment of BPV-1 is linked to a plasmid which was derived from pBR322 by the deletion of those plasmid sequences which inhibit SV40 DNA replication in eukaryotic cells (Lusky & Botchan, 1981). This vector replicates efficiently in both cell types and has been used to construct lines of BPV-1-transformed C127 cells which efficiently express the human β-globin gene from episomally replicating recombinant genomes (DiMaio *et al.*, 1982).

The SV40 replicon has also been used as the basis of episomal vector systems. These exploit the COS cells constructed by Gluzman (1981) which, because they contain functional large T-antigen and the permissivity factors required for viral DNA replication, should replicate any DNA molecule containing a functional SV40 origin of DNA replication. Myers & Tjian (1980) showed that COS cells will indeed replicate plasmid DNAs containing the SV40 origin of DNA replication on an 85 base-pair fragment. This replication is, however, relatively inefficient if the plasmid vector is pBR322 because this plasmid contains sequences which inhibit SV40 DNA replication in eukaryotic cells. Lusky & Botchan (1981) have characterized derivatives of pBR322 from which this 'poison sequence' has been deleted and SV40 origin-containing recombinants based on such vectors replicate efficiently following transfection into COS cells. Mellon *et al.* (1981) and Humphries *et al.* (1982) have used this system to study the transcription of human globin genes and have showed that they are efficiently transcribed to yield RNA indistinguishable from authentic globin mRNA.

The COS cell system has the advantage that the recombinant genome replicates to high levels, giving 200000 to 400000 copies per transfected cell (Mellon *et al.*, 1981), thus greatly facilitating analysis of the transcription of the inserted gene. However, transfection of COS cells does not lead to the establishment of stable cell lines, presumably because the accumulation of so much extra-chromosomal DNA is lethal and thus the BPV-1-based vectors appear to be the best available compromise between high levels of expression and the construction of stable cell lines.

Dominant selectable genes

The use of *E. coli* as a host for recombinant DNA molecules would not have been possible if it had not been for the availability of dominant selectable genes, most notably those encoding antibiotic resistance, which allow selection of cells which have taken up foreign DNA. Transfection of DNA into eukaryotic cells is also an inefficient process and there is thus the same need for selectable markers. The selection system which has been most widely used depends upon the tk gene of HSV and *tk*$^-$ mutant cells (for review see Scangos & Ruddle, 1981). If a cloned tk gene is co-transfected with a clone containing the gene of interest, cells selected for growth in HAT medium are found to have incorporated and to be expressing not only the tk gene

but also the non-selected marker. However, this system is restricted in its applicability because it requires *tk*⁻ mutants, the isolation of which is not a trivial proposition. Ideally, one requires selectable genes which are genetically dominant and can thus be used in any cell type.

Berg and his colleagues have recently developed two such systems in which genes of bacterial origin are carried within vectors derived from SV40. The selectable gene is expressed from the SV40 early promoter and two other segments of viral DNA are positioned at the 3′ end of the gene. One contains an intron from the SV40 early transcription unit, thus endowing the hybrid mRNA with a functional splicing site, a feature which in some (Gruss *et al.*, 1979; Gruss & Khoury, 1980) but not all (Gething and Sambrook, 1981; Gruss *et al.*, 1981*b*; Sveda and Lai, 1981; Treisman *et al.*, 1981) cases is required for the efficient expression of genes in eukaryotic cells, the other containing the polyadenylation signal from the same transcription unit. These pSV series vectors also contain a segment of pBR322 DNA including the ampicillin resistance gene and the plasmid origin of DNA replication thus allowing propagation in *E. coli* and in some cases contain a functional SV40 or polyoma virus early region, together with an origin of DNA replication, thus allowing replication in the appropriate permissive eukaryotic cells.

The first selectable marker was the *E. coli gpt* gene which encodes the enzyme xanthine–guanine phosphoribosyltransferase (XGPRT), which is the prokaryotic analogue of the mammalian enzyme hypoxanthine–guanine phosphoribosyltransferase (HGPRT). Mulligan & Berg (1980) originally cloned this gene into an SV40 late replacement vector and showed that CV-1 cells infected with the recombinant virions synthesize considerable amounts of the *E. coli* enzyme, which can be distinguished from its eukaryotic counterpart by its electrophoretic mobility and its insensitivity to inhibition by hypoxanthine. If cultured eukaryotic cells are treated with mycophenolic acid, an inhibitor of IMP dehydrogenase, they can not produce GMP unless the medium is supplemented with guanine and either hypoxanthine or adenine. The inhibition is not overcome if the medium is supplemented with adenine and xanthine because the mammalian HGPRT can not convert xanthine to XMP and thus GMP can not be produced. However, a cell expressing the *E. coli* XGPRT can utilize xanthine and escape from the mycophenolic acid inhibition, thus allowing the selection of cells expressing the *gpt* gene (Mulligan & Berg, 1981). Co-transfection followed by selection for *gpt* expression thus allows the introduction of foreign genes into any cell type.

A second, and perhaps technically more straightforward, system employs the aminoglycoside 3′-phosphotransferase gene from the *E. coli* transposon Tn5. When expressed in *E. coli*, this enzyme confers resistance to neomycin and kanamycin. Jiminez & Davies (1980) showed that a similar enzyme encoded by Tn601 will, when expressed in yeast, confer resistance to the 3′-deoxystreptamine antibiotic G418, which inhibits protein synthesis in eukaryotic cells, and thus provides a dominant selectable system. Colbere-Garapin *et al.*, (1981) showed that expression of the bacterial gene in transfected eukaryotic cells could be achieved when it was fused to the promoter and polyadenylation signal of the HSV tk gene and that cells selected for G418 resistance will incorporate and express a co-transfected DNA. Southern & Berg (1982) have described a set of pSV series vectors in which the selectable gene is that encoding the phosphotransferase and have also shown that this system provides a generally applicable dominant selectable marker. A particular feature of this system is that the same gene can be used as a selector in *E. coli*, yeast and higher eukaryotic cells.

Canaani & Berg (1982) have used pSV series vectors carrying the phosphotransferase gene to insert a human β-interferon gene into mouse and rabbit cells. They showed that the inserted gene is expressed and, as was the case with the BPV-1 recombinants discussed above, that expression can be induced by poly(I)·poly(C). Schumperli *et al.* (1982) have used the *gpt* system to insert the *E. coli* galactokinase gene into mammalian cells and, in an experiment of particular interest, have shown that expression of the bacterial enzyme can overcome the genetic defect in galactokinase-defective hamster cells.

The CAT and DHFR systems

The definition of eukaryotic promoters has turned out to be an extremely complex task. It appears that at least two classes of transcriptional control elements can be distinguished.

'Minimal' promoters contain all of the sequences required to allow the correct initiation of RNA polymerase II transcription *in vitro*; the action of such elements is highly dependent upon their position relative to the structural gene and absolutely dependent upon their orientation. Enhancers are elements which increase the level of transcription from a 'minimal' promoter *in vivo*; in some cases enhancer sequences are absolutely required for *in vivo* expression (Banerji *et al.*, 1981; Gruss *et al.*, 1981*a*; de Villiers & Schaffner, 1981; Moreau *et al.*, 1981; Levinson *et al.*, 1982; Conrad & Botchan, 1982). The striking feature of enhancers is that their action is position- and orientation-independent. These crude definitions should not be taken to imply that promoters and enhancers may not in some cases be interdigitated, or even coincident, nor that there may not be additional elements required for maximal transcription *in vivo*. However, it is clear that these two separable functions must be assayed separately.

To detect promoter function, one requires an easily assayable gene from which the normal promoter has been removed and on to which other promoters can be joined in such a way that they can be expected to drive the transcription of the assayable gene. The tk gene of HSV has been widely used for such purposes, the assay being the transformation of tk^- cells to the tk^+ phenotype. However, this assay is indirect because there are many steps between the transcription of the tk gene and the appearance of a focus of transformed cells and also because expression of the tk gene is subject to cellular regulation. Gorman *et al.* (1982*a*) have described an elegant assay system for promoter function which depends upon the gene from the transposon Tn9 which encodes chloramphenicol-acetyltransferase (CAT), the enzyme which inactivates chloramphenicol by acetylating it. This gene can be expressed from eukaryotic promoters and there is a simple and sensitive assay for enzymatic activity which, because eukaryotic cells never express this enzyme, has no background. Gorman *et al.* (1982*a*) have constructed a series of pSV series vectors carrying the CAT gene and shown that enzymic activity can be detected in a variety of eukaryotic cells following transfection of pSV2CAT in which expression is driven by the SV40 early promoter. pSV0CAT lacks a functional promoter but contains a *Hin*dIII site immediately upstream of the CAT structural gene. This vector has been used to assess the strength of a variety of eukaryotic promoters (Gorman *et al.*, 1982*a*, *b*). It is also possible that CAT expression could provide the basis of a dominant selection system but this point has not yet been tested.

The mouse *dhfr* gene has also been used as the basis for a selectable system. Subramani *et al.* (1981) have cloned mouse *dhfr* cDNA in both SV40 late replacement vectors and in pSV series plasmid vectors. Transfection of pSV2*dhfr* into DHFR-negative CHO cells allows the selection of DHFR-positive clones which by virtue of their expression of the exogenous gene can grow in the absence of thymidine, glycine and purines. Although this system is not dominant it has one considerable advantage. In cells selected for resistance to the antifolate drug methotrexate, a major cause of resistance is the synthesis of very large amounts of wild-type DHFR because of gene amplification. Schimke *et al.*, (1981) have shown that the amplification unit is very large, including considerable amounts of DNA flanking the *dhfr* gene.One might therefore expect any gene linked to *dhfr* to become amplified during selection for methotrexate resistance. Kaufman & Sharp (1982) have exploited just this strategy. They added a promoter and splicing signals to a mouse *dhfr* cDNA clone, linked this construct to the entire SV40 genome and then transfected the resultant hybrid DNA molecules into $DHFR^-$ CHO cells, selecting firstly for the expression of DHFR and secondly for methotrexate resistance. In this way they succeeded in generating a cell line in which greater than 10% of the total soluble protein is a polypeptide related to SV40 small t-antigen. Christman *et al.* (1982) and Ringold *et al.* (1982) have performed similar experiments and generated cell lines over-expressing the human hepatitis B virus surface antigen and *E. coli* XGPRT, respectively. This approach has a great deal of appeal because it does not involve complex manipulations to insert genes into viral vectors, the exogenous gene is carried in the chromosome and the result is a cell line which expresses extremely high levels of the desired protein.

Regulatable promoters

In all of the systems discussed above it would be extremely advantageous for the transcription of the cloned gene to be driven by a regulatable promoter so that expression could be switched on

or off at the investigator's convenience. Two such systems have been explored so far. Metallothioneins are metal-binding proteins, the synthesis of which is inducible at the level of transcription by heavy metal. Hamer & Walling (1982) have cloned the mouse metallothionein gene into both a SV40 virus and into a SV40 plasmid vector, transfected the resulting recombinants into monkey cells and shown that transcription of the metallothionein gene is induced 10- to 30-fold by 5 μM-$CdCl_2$. Palmiter *et al.* (1982) have produced transgenic mice carrying a hybrid gene in which HSV tk coding sequences are joined to the metallothionein promoter and have shown that Cd^{2+} will induce transcription of this in the animal. The metallothionein promoter, which has been partially characterized, thus provides a conveniently regulated control element which can be incorporated into eukaryotic vectors. The promoter present in the LTR of mouse mammary tumour virus (MMTV) is regulated by the synthetic glucocorticoid dexamethasone, apparently by direct interaction between the hormone receptor complex and sequences within the LTR (Govinden *et al.*, 1982). Lee *et al.* (1981) have shown that the expression of an exogenous gene linked to this promoter is regulated by dexamethasone but the induction ratio is low. Many experiments would be greatly facilitated by the isolation and incorporation into vectors of a eukaryotic promoter from which transcription could be regulated over several orders of magnitude.

CONCLUSIONS

The examples discussed above show clearly that the development of vectors for use in eukaryotic cells is presently proceeding very rapidly. Knowledge gained from the use of one type of vector defines the design criteria for the next type and thus many systems become obsolete before they are fully developed. Some future work can be predicted with reasonable certainty. The vast majority of future vectors will incorporate dominant selectable markers and regulatable promoters and we can expect to see vectors containing several different origins of DNA replication so that their replication can be controlled in several ways. In parallel with these refinements will occur the development of eukaryotic vectors suitable for the primary cloning of genomic DNA or cDNA. This will be of the greatest importance as it will allow the immunological or phenotypic recognition of genes by virtue of their expression in eukaryotic cells, thus facilitating the isolation of eukaryotic genes which can not be detected by the techniques available for screening prokaryotic clones. The use of such vector systems will surely be a central feature of all future work in eukaryotic molecular biology.

I am grateful to Angela Barnwell for her preparation of the manuscript. I am supported by a Career Development Award from the Cancer Research Campaign.

REFERENCES

BANERJI, J., RUSCONI, S. & SCHAFFNER, W. (1981). Expression of a β-globin gene is enhanced by remote SV40 DNA sequences. *Cell* **27**, 299–308.

BENOIST, C. & CHAMBON, P. (1981). *In vivo* sequence requirements of the SV40 early promoter region. *Nature, London* **290**, 304–310.

BRAMMAR, W. J. (1982). Vectors based on bacteriophage lambda. In *Genetic Engineering*, vol. 3, pp. 53–81. Edited by R. Williamson. New York & London: Academic Press.

CANAANI, D. & BERG, P. (1982). Regulated expression of human interferon-β gene after transduction into cultured mouse and rabbit cells. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 5166–5170.

CHRISTMAN, J. K., GERBER, M., PRICE, P. M., FLORDELLIS, C., EDELMAN, J. & ACS, G. (1982). Amplification of expression of hepatitis B surface antigen in 3T3 cells cotransfected with a dominant-acting gene and cloned viral DNA. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 1815–1819.

CHU, G. & SHARP, P. A. (1981). SV40 DNA transfection of cells in suspension: analysis of the efficiency of transcription and translation of T-antigen. *Gene* **13**, 197–202.

COLBERE-GARAPIN, F., HORODNICEANU, F., KOURILSKY, P. & GARAPIN, A-C. (1981). A new dominant hybrid selective marker for higher eukaryotic cells. *Journal of Molecular Biology* **150**, 1–14.

CONRAD, S. E. & BOTCHAN, M. R. (1982). Isolation and characterization of human DNA fragments with nucleotide sequence homologies within the simian virus 40 regulatory region. *Molecular and Cellular Biology* **2**, 949–965.

CORDEN, J., WASYLYK, B., BUCKWALDER, A., SASSONE-CORSI, P., KEDINGER, C. & CHAMBON, P. (1980). Promoter sequences of eukaryotic protein-coding genes. *Science* **209**, 1406–1414.

DE VILLIERS, J. & SCHAFFNER, W. (1981). A small segment of polyoma virus DNA enhances the expression of a cloned β-globin gene over a distance of 1400 base pairs. *Nucleic Acids Research* **9**, 6251–6262.

DIMAIO, D., TREISMAN, R. & MANIATIS, T. (1982). Bovine papillomavirus vector that propagates as a plasmid in both mouse and bacterial cells. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 4030–4034.

ELDER, J. T., SPRITZ, R. A. & WEISSMAN, S. M. (1981). Simian virus 40 as a eukaryotic cloning vehicle. *Annual Review of Genetics* **15**, 295–340.

GETHING, M-J. & SAMBROOK, J. (1981). Cell-surface expression of influenza haemagglutinin from a cloned DNA copy of the RNA gene. *Nature, London* **293**, 620–625.

GLUZMAN, Y. (1981). SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* **23**, 175–182.

GLUZMAN, Y. (1982). *Eukaryotic Viral Vectors*. New York: Cold Spring Harbor Laboratory.

GOFF, S. P. & BERG, P. (1976). Construction of hybrid viruses containing SV40 and λ phage DNA segments and their propagation in cultured monkey cells. *Cell* **9**, 695–705.

GOFF, S. P. & BERG, P. (1979). Construction, propagation and expression of simian virus 40 recombinant genomes containing the *Escherichia coli* gene for thymidine kinase and a *Saccharomyces cerevisiae* gene for tyrosine transfer RNA. *Journal of Molecular Biology* **133**, 359–383.

GOLDBERG, M. (1978). *Sequence analysis of* Drosophila *histone genes*. Ph.D. thesis, Stanford University Medical School.

GORMAN C. M., MOFFAT, L. F. & HOWARD, B. H. (1982*a*). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Molecular and Cellular Biology* **2**, 1044–1051.

GORMAN, C. M., MERLINO, G. T., WILLINGHAM, M. C., PASTAN, I. & HOWARD, B. H. (1982*b*). The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA mediated transfection. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 6777–6781.

GOVINDEN, M. V., SPIESS, E. & MAJORS, J. (1982). Purified glucocorticoid receptor-hormone complex from rat liver cytosol binds specifically to cloned mouse mammary tumor virus long terminal repeats *in vitro*. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 5157–5161.

GRAHAM, F. L. & VAN DER EB, A. J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**, 456–467.

GRAHAM, F. L., SMILEY, J., RUSSELL, W. C. & NAIRN, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *Journal of General Virology* **36**, 59–72.

GRODZICKER, T. & KLESSIG, D. F. (1980). Expression of unselected adenovirus genes in human cells co-transformed with the HSV-1 tk gene and adenovirus 2 DNA. *Cell* **21**, 453–463.

GRUSS, P. & KHOURY, G. (1980). Rescue of a splicing defective mutant by insertion of an heterologous intron. *Nature, London* **286**, 634–637.

GRUSS, P. & KHOURY, G. (1981). Expression of a simian virus 40–rat preproinsulin recombinant in monkey kidney cells: use of preproinsulin RNA processing signals. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 133–137.

GRUSS, P., LAI, C-J., DHAR, R. & KHOURY, G. (1979). Splicing as a requirement of biogenesis of functional 16S mRNA of simian virus 40. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4317–4321.

GRUSS, P., DHAR, R. & KHOURY, G. (1981*a*). Simian virus 40 tandem repeated sequences as an element of the early promoter. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 943–947.

GRUSS, P., EFSTRATIADIS, A., KARATHAMASIS, S., KONIG, M. & KHOURY, G. (1981*b*). Synthesis of stable unspliced mRNA from an intronless simian virus 40–rat preproinsulin gene recombinant. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 6091–6095.

HAMER, D. H. & LEDER, P. (1979*a*). Expression of the chromosomal mouse β-maj-globin gene cloned in SV40. *Nature, London* **281**, 35–40.

HAMER, D. H. & LEDER, P. (1979*b*). SV40 recombinants carrying a functional RNA splice junction and polyadenylation site from the chromosomal mouse β-maj-globin gene. *Cell* **17**, 737–747.

HAMER, D. H. & LEDER, P. (1979*c*). Splicing and the formation of stable RNA. *Cell* **18**, 1299–1302.

HAMER, D. H. & WALLING, M-J. (1982). Regulation *in vivo* of a cloned mammalian gene: cadmium induces the transcription of a mouse metallothionein gene in SV40 vectors. *Journal of Molecular and Applied Genetics* **1**, 273–288.

HAMER, D. H., DAVOLI, D., THOMAS, C. A. & FAREED, G. C. (1977). Simian virus 40 carrying an *Escherichia coli* suppressor gene. *Journal of Molecular Biology* **112**, 155–182.

HAMER, D. H., SMITH, K. D., BOYER, S. H. & LEDER, P. (1979). SV40 recombinants carrying rabbit β-globin gene coding sequences. *Cell* **17**, 725–735.

HAMER, D. H., KAEHLER, M. & LEDER, P. (1980). A mouse globin gene promoter is functional in SV40. *Cell* **21**, 697–708.

HARTMAN, J. R., NAYAK, D. P. & FAREED, G. C. (1982). Human influenza virus hemagglutinin is expressed in monkey cells using simian virus 40 vectors. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 233–237.

HUMPHRIES, R. K., LEY, T., TURNER, P., MOULTON, A. D. & NIENHUIS. A. W. (1982). Differences in human α-, β- and δ-globin gene expression in monkey kidney cells. *Cell* **30**, 173–183.

JIMINEZ, A. & DAVIES, J. (1980). Expression of a transposable antibiotic resistance element in *Saccharomyces*. *Nature, London* **287**, 869–871.

JONES, N. & SHENK, T. (1978). Isolation of deletion and substitution mutants of adenovirus type 5. *Cell* **13**, 181–188.

JONES, N. & SHENK, T. (1979). Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. *Cell* **17**, 683–689.

KAUFMAN, R. J. & SHARP, P. A. (1982). Amplification and expression of sequences cotransfected with a modular dihydrofolate reductase complementary DNA gene. *Journal of Molecular Biology* **159**, 601–621.

LAW, M-F., LOWY, D. R., DVORETZKY, I. & HOWLEY, P. M. (1981). Mouse cells transformed by bovine papillomavirus contain only extrachromosomal viral DNA sequences. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 2727–2731.

LEE, F., MULLIGAN, R., BERG, P. & RINGOLD, G. (1981). Glucocorticoids regulate expression of dihydrofolate reductase cDNA in mouse mammary tumour virus chimaeric plasmids. *Nature, London* **294**, 228–232.

LEVINSON, B., KHOURY, G., VANDE WOUDE, G. & GRUSS, P. (1982). Activation of SV40 genome by 72 base-pair tandem repeats of Moloney sarcoma virus. *Nature, London* **295**, 568–572.

LOWY, D. R., DVORETZKY, I., SHOBER, R., LAW, M-F., ENGEL, L. & HOWLEY, P. M. (1980). *In vitro* tumorigenic transformation by a defined sub-genomic fragment of bovine papilloma virus. *Nature, London* **287**, 72–74.

LUSKY, M. & BOTCHAN, M. (1981). Inhibitory effect of specific pBR322 DNA sequences upon SV40 replication in simian cells. *Nature, London* **293**, 79–81.

MATHIS, D. J. & CHAMBON, P. (1981). The SV40 early region TATA box is required for accurate *in vitro* initiation of transcription. *Nature, London* **290**, 310–315.

MELLON, P., PARKER, V., GLUZMAN, Y. & MANIATIS, T. (1981). Identification of DNA sequences required for transcription of the human α_1-globin gene in a new SV40 host–vector system. *Cell* **27**, 279–288.

MOAR, M. H., CAMPO, M. S., LAIRD, H. M. & JARRETT, W. F. H. (1981*a*). Unintegrated viral DNA sequences in a hamster tumor induced by bovine papillomavirus. *Journal of Virology* **39**, 945–949.

MOAR, M. H., CAMPO, M. S., LAIRD, H. & JARRETT, W. F. H. (1981*b*). Persistence of non-integrated viral DNA in bovine cells transformed *in vitro* by bovine papillomavirus type 2. *Nature, London* **293**, 749–751.

MOREAU, P., HEN, R., WASYLYK, B., EVERETT, R., GAUB, M. P. & CHAMBON, P. (1981). The SV40 72 base-pair repeat has a striking effect on gene expression both in SV40 and other chimeric recombinants. *Nucleic Acids Research* **9**, 6047–6068.

MORIARTY, A. M., HOYER, B. H., SHIH, J. W-K., GENN, J. L. & HAMER, D. H. (1981). Expression of the hepatitis B virus surface antigen gene in cell culture by using a simian virus 40 vector. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 2606–2610.

MULLIGAN, R. C. & BERG, P. (1980). Expression of a bacterial gene in mammalian cells. *Science* **209**, 1422–1427.

MULLIGAN, R. C. & BERG, P. (1981). Selection for animal cells that express the *Escherichia coli* gene coding for xanthine–guanine phosphoribosyltransferase. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 2072–2076.

MULLIGAN, R. C., HOWARD, B. H. & BERG, P. (1979). Synthesis of rabbit β-globin in cultured monkey kidney cells following infection with a SV40-β-globin recombinant genome. *Nature, London* **277**, 108–114.

MYERS, R. M. & TJIAN, R. (1980). Construction and analysis of simian virus 40 origins defective in tumor antigen binding and DNA replication. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 6491–6495.

MYERS, R. M., RIO, D. C., ROBBINS, A. K. & TJIAN, R. (1981). SV40 gene expression is modulated by the cooperative binding of T-antigen to DNA. *Cell* **25**, 373–384.

NUNBERG, J. G., KAUFMAN, R. J., CHANG, A. C. Y., COHEN, S. N. & SCHIMKE, R. T. (1980). Structure and genomic organisation of the mouse dihydrofolate reductase gene. *Cell* **19**, 355–364.

PALMITER, R. D., CHEN, H. Y. & BRINSTER, R. L. (1982). Differential regulation of metallothionein-thymidine kinase fusion genes in transgenic mice and their offspring. *Cell* **29**, 701–710.

PANICALI, D. & PAOLETTI, E. (1982). Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 4927–4931.

RASSOULZADEGAN, M., BINETRUY, B. & CUZIN, F. (1982). High frequency of gene transfer after fusion between bacteria and eukaryotic cells. *Nature, London* **295**, 257–259.

RIGBY, P. W. J. (1982). Expression of cloned genes in eukaryotic cells using vector systems derived from viral replicons. In *Genetic Engineering*, vol. 3, pp. 83–141. Edited by R. Williamson. New York & London: Academic Press.

RINGOLD, G., DIECKMANN, B. & LEE, F. (1982). Co-expression and amplification of dihydrofolate reductase cDNA and the *Escherichia coli* XGPRT gene in Chinese hamster ovary cells. *Journal of Molecular and Applied Genetics* **1**, 165–175.

SARVER, N., GRUSS, P., LAW, M-F., KHOURY, G. & HOWLEY, P. M. (1981*a*). Bovine papilloma virus deoxyribonucleic acid: a novel eucaryotic cloning vector. *Molecular and Cellular Biology* **1**, 486–496.

SARVER, N., GRUSS, P., LAW, M-F., KHOURY, G. & HOWLEY, P. M. (1981*b*). Rat insulin gene covalently linked to bovine papillomavirus DNA is expressed in transformed mouse cells. In *Developmental Biology Using Purified Genes*. ICN–UCLA Symposia on Molecular and Cellular Biology vol. 23, pp. 547–556. Edited by D. Brown and C. R. Fox. New York & London: Academic Press.

SCANGOS, G. & RUDDLE, F. H. (1981). Mechanisms and applications of DNA-mediated gene transfer in mammalian cells; a review. *Gene* **14**, 1–10.

SCHAFFNER, W. (1980). Direct transfer of cloned genes from bacteria to mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 2163–2167.

SCHIMKE, R. T., BROWN, P. C., KAUFMAN, R. J., McGROGAN, M. & SLATE, D. L. (1981). Chromosomal and

extrachromosomal localization of amplified dihydrofolate reductase genes in cultured mammalian cells. *Cold Spring Harbor Symposia on Quantitative Biology* **45**, 785–798.

SCHUMPERLI, D., HOWARD, B. H. & ROSENBERG, M. (1982). Efficient expression of *Escherichia coli* galactokinase gene in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 257–261.

SHIMOTOHNO, K. & TEMIN, H. M. (1981). Formation of infectious progeny virus after insertion of herpes simplex thymidine kinase gene into DNA of an avian retrovirus. *Cell* **26**, 67–77.

SHIMOTOHNO, K. & TEMIN, H. M. (1982). Loss of intervening sequences in genomic mouse α-globin DNA inserted in an infectious retrovirus vector. *Nature, London* **299**, 265–268.

SOLNICK, D. (1981). Construction of an adenovirus-SV40 recombinant producing SV40 T-antigen from an adenovirus late promoter. *Cell* **24**, 135–143.

SOUTHERN, P. J. & BERG, P. (1982). Transformation of mammalian cells to antibiotic resistance with a bacterial gene under the control of the SV40 early region promoter. *Journal of Molecular and Applied Genetics* **1**, 327–341.

SPAETE, R. R. & FRENKEL, N. (1982). The herpes simplex virus amplicon: a new eukaryotic defective-virus cloning-amplifying vector. *Cell* **30**, 295–304.

STOW, N. (1981). Cloning of a DNA fragment from the left-hand terminus of the adenovirus type 2 genome and its use in site-directed mutagenesis. *Journal of Virology* **37**, 171–180.

STOW, N. D. (1982*a*). The infectivity of adenovirus genomes lacking DNA sequences from their left-hand termini. *Nucleic Acids Research* **10**, 5105–5119.

STOW, N. D. (1982*b*). Localization of an origin of DNA replication within the TRs/IRs repeated region of the herpes simplex virus type 1 genome. *EMBO Journal* **1**, 863–867.

SUBRAMANI, S., MULLIGAN, R. & BERG, P. (1981). Expression of the mouse dihydrofolate reductase complementary deoxyribonucleic acid in simian virus 40 vectors. *Molecular and Cellular Biology* **1**, 854–864.

SVEDA, M. M. & LAI, C-J. (1981). Functional expression in primate cells of cloned DNA coding for the hemagglutinin surface glycoprotein of influenza virus. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 5488–5492.

TABIN, C. J., HOFFMANN, J. W., GOFF, S. P. & WEINBERG, R. A. (1982). Adaptation of a retrovirus as a eucaryotic vector transmitting the herpes simplex virus thymidine kinase gene. *Molecular and Cellular Biology* **2**, 426–436.

THUMMEL, C., TJIAN, R. & GRODZICKER, T. (1981). Expression of SV40 T-antigen under control of adenovirus promoters. *Cell* **23**, 825–836.

TOOZE, J. (1981). *Molecular Biology of Tumor Viruses. Part 2: DNA Tumor Viruses*, 2nd Edn. New York: Cold Spring Harbor Laboratory.

TREISMAN, R., NOVAK, U., FAVALORO, J. & KAMEN, R. (1981). Transformation of rat cells by an altered polyoma virus genome expressing only the middle-T protein. *Nature, London* **292**, 595–600.

WEI, C-M., GIBSON, M., SPEAR, P. G. & SCOLNICK, E. M. (1981). Construction and isolation of a transmissible retrovirus containing the *src* gene of Harvey murine sarcoma virus and the thymidine kinase gene of herpes simplex virus type 1. *Journal of Virology* **39**, 935–944.

WEISS, R. A., TEICH, N. M., VARMUS, H. & COFFIN, J. M. (1982). *Molecular Biology of Tumor Viruses. Part 3: RNA Tumor Viruses*, 2nd edn. New York: Cold Spring Harbor Laboratory.

WILLIAMS, B. G. & BLATTNER, F. R. (1980). Bacteriophage lambda vectors for DNA cloning. In *Genetic Engineering: Principles and Methods*, vol. 2, pp. 201–281. Edited by J. K. Setlow & A. Hollaender. New York: Plenum Press.

ZINN, K., MELLON, P., PTASHNE, M. & MANIATIS, T. (1982). Regulated expression of an extrachromosomal human β-interferon gene in mouse cells. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 4897–4901.