CHAPTER 11

Papillomavirus Cloning Vectors

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I. INTRODUCTION

The papillomaviruses are proving to be valuable tools for transferring genes into living mammalian cells because the viral DNA can be used to establish permanent cell lines containing the transferred genes as unrearranged, extrachromosomal DNA molecules. This use of these viruses may help elucidate the function and regulation of eukaryotic genes and may enable the production of gene products that would otherwise be difficult to obtain. Bovine papillomavirus type 1 is the only papillomavirus to be exploited so far as a cloning vector, but it is likely that similar vectors will also be derived from DNA of other papillomaviruses.

A number of techniques have been developed for introducing purified genes into cells in tissue culture. The gene of interest is often covalently joined to a cloning vehicle (or vector) to facilitate identification of cells that have taken up the DNA or to ensure its propagation or expression. Cloning vectors have been developed from a number of animal viruses including SV40 and polyoma virus, adenoviruses, herpesviruses, poxviruses, and retroviruses (Gluzman, 1982). Infection by most of these viruses culminates in cell death, so it is often not possible to establish permanent cell lines for long-term biochemical and genetic manipulation. Methods that do generate stable cell lines almost invariably result in rearrangement of the transferred DNA and its joining to diverse uncharacterized segments of DNA (Perucho *et al.*, 1980). Consequently, quantitative com-

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N. P. Salzman et al. (eds.), *The Papovaviridae*

parisons of gene expression in different cell lines are difficult to make. In an attempt to circumvent these problems, a new class of eukaryotic cloning vectors has been developed from BPV-1 DNA. This chapter describes these vectors, summarizes the results obtained by using them, and discusses their potential applications.

II. TRANSFORMATION BY BOVINE PAPILLOMAVIRUS

BPV-1 DNA can oncogenically transform susceptible rodent fibroblasts in culture. This process and the properties of the transformed cells are discussed in detail in an earlier chapter in this volume, but the relevant points will be reiterated here. (1) About 1 to 2 weeks after purified viral DNA is applied to certain lines of mouse (C127, NIH 3T3) or rat (FR 3T3) fibroblasts, dense foci of cells appear on the monolayer of normal cells. Viral DNA cloned in *E. coli* retains this focus-forming ability. (2) An intact, unit-length viral genome is not required for transformation because a 5500-bp subgenomic fragment of viral DNA is sufficient to transform cells. (3) Papillomavirus transformation does not result in cell death; rather, the cells stably acquire a set of altered growth properties including anchorage independence and the ability to form tumors in nude mice. (4) The viral DNA does not appear to integrate into the cellular genome. Instead, up to several hundred apparently identical copies of the viral DNA are usually maintained as circular DNA molecules in the nuclei of transformed cells. DNA of BPV-2 and BPV-4 (Moar *et al.*, 1981; Campo and Spadidos, 1983), the cottontail rabbit papillomavirus (Watts *et al.*, 1983), the deer fibromavirus (Groff *et al.*, 1983), the European elk papillomavirus (Stenlund *et al.*, 1983b), and human papillomavirus types 1 and 5 (Watts *et al.*, 1984) have also been reported to transform rodent cells in an analogous manner.

Most gene transfer experiments using BPV-1 vectors take the following form. Recombinant molecules are constructed that contain the gene of interest, a transforming segment of BPV-1 DNA, and a bacterial replicon. The availability of the complete BPV-1 DNA sequence greatly facilitates the construction of these molecules (Chen *et al.*, 1982). The prokaryotic sequences are included to allow the easy preparation of large amounts of the recombinant DNA for subsequent steps. The DNA is then transferred as a calcium phosphate precipitate (usually with carrier DNA) or via protoplast fusion to susceptible rodent cells growing on plastic. Because papillomaviruses do not form infectious progeny in tissue culture, it has not been possible to encapsidate recombinant molecules in BPV-1 virions for efficient introduction into cells. About 2 weeks after exposure to DNA, individual transformed foci or colonies can be isolated and expanded into cell lines for analysis. Although only very few cells exposed to DNA or plasmid-containing bacteria become stably transformed (often less than 10^{-4}), it is possible to derive cell lines consisting

entirely of transformed cells which can be propagated indefinitely or frozen in media containing 8% dimethyl sulfoxide. There have been no reports of transient expression experiments in which expression of a BPV-1-linked gene is assayed within a few days of DNA transfer. Technical details concerning the generation and analysis of BPV-1-transformed cell lines can be found in earlier brief reviews on BPV-1 cloning vectors (Howley *et al.*, 1983; Sarver *et al.*, 1983) and in the references cited in this chapter. Recently, Campo (1985) has compiled a number of relevant protocols and numerous restriction endonuclease cleavage maps of cloning vectors containing BPV-1 DNA.

In transformed cells, the transferred, BPV-1-linked DNA is usually present as an extrachromosomal DNA molecule. This is the primary attraction to using BPV-1 as a vector because it allows the establishment of permanent cell lines which contain the transferred gene in a stable, completely defined sequence environment. The advantages of studying transferred genes in this configuration are listed in Table I. It is clear that understanding the factors which influence either the efficiency of focus formation by BPV-1 plasmids or the extrachromosomal maintenance of these plasmids is crucial for the successful design of vectors. The protocols used for DNA transfer and the structure of the BPV-1 plasmids both affect transformation efficiency. NIH 3T3 cells are more readily transformed by BPV-1 DNA than are C127 cells (Lowy *et al.*, 1980), but the latter cell line has been used in most gene transfer experiments because transformed C127 cells give rise to foci which are more distinct. Primary hamster embryo cells can also be transformed by BPV-1 DNA (Morgan and Meinke, 1980), but attempts to transform human cells with BPV-1 or BPV-1 DNA have been unsuccessful (Black *et al.*, 1963; Dvoretzky *et al.*, 1980). It is the general experience that as C127 cells are passaged in culture, they become refractory to DNA-mediated transformation and are more likely to undergo spontaneous transformation. The batch of serum used for cell culture can also greatly affect the efficiency with which transformed foci are generated (Giri *et al.*, 1983), and in one set of experiments the omission of carrier DNA abolished the focus-forming activity of one of two recombinant plasmids tested (Matthias *et al.*, 1983). Although DNA has been transferred as a calcium phosphate precipitate in most experiments, it

TABLE I. Advantages of Cloning Vectors That Propagate as Plasmids

Plasmid DNA can readily be purified away from high-molecular-weight cellular DNA
Minichromosomes containing the gene of interest can readily be purified away from cellular chromosomes
DNA on all copies of a particular plasmid is in a uniform sequence environment. This may allow uniform expression levels of genes on these plasmids
The potential problem of integration of transferred DNA into inactive regions of cellular DNA is eliminated

has been reported for several plasmids that foci are more readily obtained following protoplast fusion (Schaffner, 1980; Binetruy *et al.*, 1982).

Because much of the molecular biology of BPV-1 is not understood in detail, the construction of stable BPV-1 cloning vectors is largely a matter of trial-and-error. Nevertheless, a few general principles of vector design can be outlined (see Fig. 1 for schematic diagram of an idealized BPV-1 cloning vector). The DNA segment inserted into the vector must not interrupt an essential gene or regulatory signal of the viral DNA. The finding that the 5500-bp *Hin*dIII to *Bam*HI restriction fragment of BPV-1 DNA is sufficient to transform cells indicated that this fragment may be a suitable BPV-1 vector (Lowy *et al.*, 1980; Sarver *et al.*, 1981). The realization that transformation by the 5500-bp fragment is facilitated by the remaining portion of the viral genome or by certain segments of cellular DNA suggests that one of these facilitatory segments should be included in BPV-1 vectors (DiMaio *et al.*, 1982; Sarver *et al.*, 1982; Kushner *et al.*, 1982; Karin *et al.*, 1983). For the full-length viral genome linked to bacterial plasmid DNA, the *Bam*HI rather than the *Hin*dIII permutation

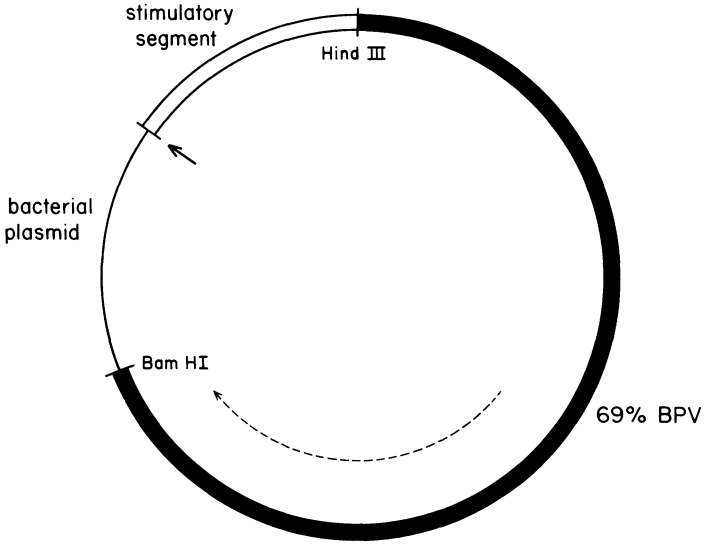

FIGURE 1. Schematic diagram of an idealized BPV-1 cloning vector. The circle represents a circular, double-stranded DNA molecule. The dark line represents the 5500-bp *Hin*dIII to *Bam*HI restriction fragment of BPV-1 DNA which is sufficient to transform mouse cells. The direction of transcription of this fragment in mouse cells is indicated by the dashed arrow. The double line represents a segment of DNA which stimulates mouse cell transformation by the BPV-1 DNA fragment. Such segments include the remaining 2500 bp of the viral genome and any of a number of DNA fragments from cellular genomes. The single line represents a bacterial replicon containing sequences that allow selection and amplification of the plasmid in bacteria. Additional DNA segment can be inserted at the position indicated by the solid arrow. Vectors of this general design include pBPV-142-6 and pBPV-BV1 (Sarver *et al.*, 1982; Zinn *et al.*, 1983).

seems preferable because it has been reported that the former is more stable (Sarver *et al.*, 1982).

The influence of linkage of BPV-1 DNA to bacterial plasmids deserves further comment because there are conflicting reports in the literature. A number of investigators have observed that the efficiency of focus induction by BPV-1 DNA is markedly reduced by covalent linkage to the bacterial plasmid pBR322 (Lowy *et al.*, 1980; Sarver *et al.*, 1981, 1982; DiMaio *et al.*, 1982; Binetruy *et al.*, 1982; Kushner *et al.*, 1982; Richards *et al.*, 1984). One group finds that this inhibition remains when pBR322 is replaced by pML2 (Binetruy *et al.*, 1982), a derivative of pBR322 lacking specific sequences which inhibit replication of linked SV40 DNA in monkey cells (Lusky and Botchan, 1981), but others find that insertion of the full-length *Bam*HI-cut BPV-1 genome into this deleted plasmid is compatible with efficient focus formation (Sarver *et al.*, 1982). In fact, other groups see no inhibition by bacterial plasmid sequences (Campo and Spandidos, 1983; Giri *et al.*, 1983). Numerous factors could account for these discrepancies. These include differences in viral isolates, host mammalian cells, transformation or cell culture protocols, DNA modifications introduced during propagation in different bacterial hosts, and exact bacterial plasmid sequences present in the recombinants. Since a systematic evaluation of the effect of these parameters has not been reported, it seems prudent to follow precisely the protocols of the laboratory from which each particular vector originated. In any case, a number of recombinant plasmids have been constructed which under certain relatively well-defined conditions efficiently give rise to transformed foci and which are maintained with minimal DNA rearrangement in the transformed cells.

III. DEVELOPMENT OF BPV-1 VECTORS

Following initial reports that bacterial plasmid sequences inhibit transformation by BPV-1 DNA, early gene transfer experiments using BPV-1 included cleavage of the recombinant plasmid with restriction endonucleases to dissociate the bacterial sequences from the DNA to be transferred. BPV-1 was first employed as a cloning vector by Sarver *et al.* (1981) who used the 5500-bp fragment of BPV-1 DNA to transfer the rat preproinsulin I gene into C127 cells. Prior to transformation, pBR322 sequences which had been used to amplify this DNA in bacteria were removed. Cells that had acquired the transferred DNA were identified on the basis of their ability to form morphologically transformed foci, and all 48 cell lines tested secreted rat proinsulin into the culture medium. These experiments established several important points about using BPV-1 as a cloning vector. In addition to demonstrating that BPV-1-induced morphological transformation could be used to select cells containing transferred genes, they also indicated that a number of appropriate eukaryotic cell-specific posttranslational modifications could occur in these

cells. Importantly, the DNA was maintained as a multicopy plasmid with apparently no integration in the transformants, demonstrating that DNA linked to BPV-1 DNA is also propagated as a plasmid. In many of the cell lines, the DNA had undergone different rearrangements including acquisition of uncharacterized segments of DNA. This variability in DNA structure among different cell lines may have accounted for the variability that was also observed in gene expression. Similar experiments have since been performed with a number of BPV-1-linked genes excised from bacterial plasmid sequences.

There are three reasons why it is unsatisfactory to separate bacterial plasmid sequences from BPV-1 DNA prior to transformation. First, the requirement for enzymatic cleavage places constraints on acceptable locations of restriction endonuclease recognition sites in the recombinant plasmids. Second, linear BPV-1 DNA often undergoes rearrangements at its ends during recircularization in cells. Finally, the separation of eukaryotic and prokaryotic replicons prevents the facile transfer of plasmids between bacteria and mouse cells. These problems were overcome to a large extent by the second generation of BPV-1 vectors, plasmids which transformed cells without prior removal of bacterial plasmid sequences. A stable prokaryotic/eukaryotic plasmid replicon containing BPV-1 and bacterial plasmid DNA was first reported by DiMaio *et al.* (1982) who found that a 7600-bp DNA fragment containing the human β-globin gene caused a several hundred-fold stimulation of focus formation in C127 cells by the 5500-bp segment of BPV-1 DNA inserted in a bacterial plasmid. The transferred DNA in the transformants was often present initially as about 30 unrearranged, monomeric plasmids per cell, although rearrangements occasionally occurred with continued passage of the cell. Plasmids could be reestablished in *E. coli* by transforming competent bacteria with low-molecular-weight DNA from mouse cells and selecting for ampicillin-resistant colonies. The methylation pattern of the DNA giving rise to these colonies was consistent with its having replicated in animal cells, indicating that it was not contaminating DNA, and colonies generated in this manner contained plasmid DNA indistinguishable from the molecules originally used to transform mouse cells (Fig. 2). These experiments demonstrated that it was possible to introduce an intact plasmid into mouse cells where it was stably maintained as a monomeric, circular DNA molecule, and to reestablish the plasmid in bacteria without further enzymatic manipulation.

The mechanism by which the globin DNA stimulates transformation is unknown, but analysis of mutants with deletions in the globin region indicate that 2700 bp of human DNA is sufficient to cause this effect (Zinn *et al.*, 1983). Transformation of C127 cells by the subgenomic segment of BPV-1 DNA is stimulated by other cellular DNA fragments including the rat and human growth hormone genes (Kushner *et al.*, 1982; Sarver *et al.*, 1982), human metallothionein II_A gene (Karin *et al.*, 1983), an intergenic DNA segment from the rat fibrinogen gene cluster (Sarver

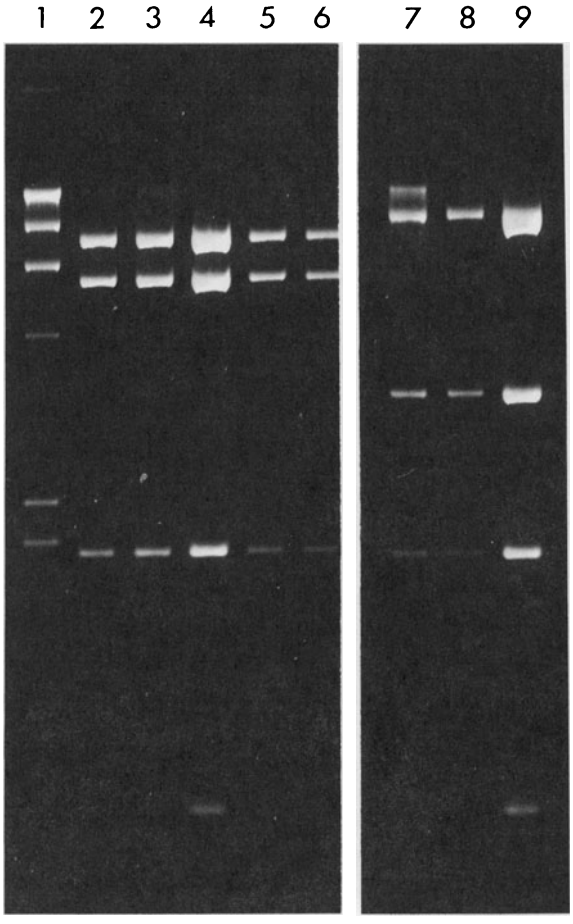

FIGURE 2. Recovery of BPV-1 plasmids from transformed mouse cells. C127 cells were transformed with pBPV-β1 and pBPV-β3 (DiMaio *et al.*, 1982). Low-molecular-weight DNA was isolated from cell lines established from individual foci of transformed cells and transferred into bacteria. DNA of the input plasmids was compared to DNA from individual ampicillin-resistant bacterial colonies after digestion with *Bam*HI and agarose gel electrophoresis. Lane 1: molecular weight markers. Lanes 2, 3, 5, 6: plasmids rescued from mouse cells transformed with pBPV-β3. Lane 4: pBPV-β3. Lanes 7, 8: plasmids rescued from mouse cells transformed with pBPV-β1. Lane 9: pBPV-β1.

et al., 1982), and the rat preproinsulin gene (Sarver *et al.*, 1982). Except for the human metallothionein gene which also stimulates transformation of NIH 3T3 cells, it is not known if these sequences are active in other cell types. Inactive DNA fragments include the human β-interferon gene (Zinn *et al.*, 1982), the rat γ-fibrinogen gene (Sarver *et al.*, 1983), the mouse metallothionein I gene (Sarver *et al.*, 1983), a human class I histocompatibility antigen heavy chain gene (DiMaio *et al.*, 1984), and the human metallothionein I gene (Richards *et al.*, 1984). Cells can also be

efficiently transformed by certain full-length BPV-1–pML2 plasmids without additional DNA segments (Sarver *et al.*, 1982). These plasmids are also stably maintained in the transformants, and they can be rescued in bacteria. It should be possible to derive useful vectors from any of these plasmids unless the insertion of additional DNA interferes with focus formation or plasmid stability. Stable plasmid vectors containing either the full-length *Bam*HI-generated viral genome or the 5500-bp transforming fragment plus a stimulatory segment of cellular DNA have been successfully used to transfer additional DNA segments (Law *et al.*, 1983; Zinn *et al.*, 1983; Schenborn *et al.*, 1985; Sambrook *et al.*, 1985).

The utility of such vectors would probably be greatly increased if the recombinant plasmids contained selectable markers in addition to the BPV-1 transforming function. Experiments using the herpes simplex virus thymidine kinase gene (Lusky *et al.*, 1983; Sekiguchi *et al.*, 1983), the *E. coli* gpt gene (Law *et al.*, 1982; Giri *et al.*, 1983), or a mouse dihydrofolate reductase gene (Breathnach, 1984) were unsatisfactory because the BPV-1 DNA in cells containing the selected gene had frequently undergone rearrangement and/or integration into cellular DNA. More recently, both the neomycin analogue G418 and heavy metals have been used to select for extrachromosomal BPV-1-linked genes. Most mammalian cells are killed by G418, but resistance can be conferred by a gene isolated from Tn5, a bacterial transposon (Colbere-Garapin *et al.*, 1981; Southern and Berg, 1982). Several groups have constructed recombinant plasmids containing BPV-1 DNA and the G418 resistance gene under the control of eukaryotic regulatory signals and have demonstrated that after transfer of these recombinants and selection of drug-resistant cells, the DNA is maintained as a multicopy plasmid in the resistant mouse or rat cells (Law *et al.*, 1983; Matthias *et al.*, 1983; Lusky and Botchan, 1984; Meneguzzi *et al.*, 1984). Some of these plasmids are relatively unstable, but there appears to be less plasmid rearrangement in cell lines selected for drug resistance rather than for morphological transformation (Matthias *et al.*, 1983; Meneguzzi *et al.*, 1984). Similarly, resistance to the toxic heavy metals cadmium and zinc can be conferred by BPV-1-linked, extrachromosomal cloned mouse or human metallothionein genes (Karin *et al.*, 1983; Pavlakis and Hamer, 1983a,b).

If insertion of additional DNA is still compatible with selection and does not result in plasmid instability, these vectors may allow the introduction of extrachromosomal, BPV-1-linked genes into cell types not susceptible to oncogenic transformation by the papillomaviruses. Moreover, Lusky and Botchan (1984) have found that autonomous replication can proceed in the absence of some BPV-1 functions required for cell transformation. One can thus envision a class of transformation-defective BPV-1 vectors that still propagate as plasmids and can be selected biochemically. For many studies of gene expression, it may be preferable to use such vectors to eliminate the effects of oncogenic transformation.

IV. EXPRESSION OF GENES CLONED ON BPV-1

The ultimate goal of most gene transfer experiments is the examination of the expression of the transferred gene in the host cell. The nucleotide sequences controlling the structure and expression of a gene product can be identified and characterized by transferring genes carrying defined mutations. The effect of a foreign gene on the cell can also be assessed, as can the effects of an altered gene product or altered levels of a normal gene product. BPV-1 vectors are especially suited for studying genes isolated from mammals because the rodent host cells contain much of the cellular machinery necessary for accurate expression of these genes. In fact, not only are numerous posttranscriptional and posttranslational modifications correctly carried out in BPV-1-transformed cells (Some of which are listed in Table II), but certain levels of gene control are also retained in the transformants.

Using BPV-1 vectors to study gene regulation is not without its hazards. Events occurring in cells oncogenically transformed by BPV-1 may not necessarily reflect the situation in normal cells. For example, it is known that transforming proteins of other DNA viruses can activate transferred cellular and viral genes (Imperiale *et al.*, 1983; Green *et al.*, 1983). The multicopy nature of the BPV-1 system poses additional com-

TABLE II. Posttranslational Modifications Reported for Products of BPV-1-Linked Genes

Proteolytic cleavage
Rat preproinsulin I
Human growth hormone
Glycosylation
Hepatitis B virus surface antigen
Vesicular stomatitis virus G protein
Influenza virus hemagglutinin
Membrane insertion
Vesicular stomatitis virus G protein
Human histocompatibility antigen heavy chain
Influenza virus hemagglutinin
Secretion
Rat preproinsulin I
Hepatitis B virus surface antigen
Human β-interferon
Human growth hormone
Vesicular stomatitis virus G protein (mutant)
Minute virus of mice capsid proteins
Influenza virus hemagglutinin (mutant)
Assembly in macromolecular aggregates
Hepatitis B virus surface antigen
Human histocompatibility antigen heavy chain
Minute virus of mice capsid proteins

plications because the proportion of transcriptionally active genomes has not been established. Therefore, transcriptional induction could be due either to an increased rate of transcription of each gene or to recruitment of more templates into the active fraction. Although the assumption is made that the expression detected results from transcription of extrachromosomal templates, in most of these gene transfer experiments it has not been ruled out that the transformants contain a small amount of integrated exogenous DNA. It is particularly difficult to distinguish between large oligomeric plasmids and tandemly arrange integrated molecules. It is conceivable that in some experiments a small number of very active, integrated or rearranged templates may account for the observed expression.

The sections below describe the experience with a number of specific genes introduced into cells on BPV-1 vectors.

A. Human β-Globin Gene

The globin genes code for the polypeptide subunits of hemoglobin, the major soluble protein and oxygen-carrying component of red blood cells. Because of their important physiological role and because of the ease of obtaining large amounts of purified material, the globin genes and their products have been intensively studied for many years. The globin DNA fragment which stimulates transformation by the 5500-bp fragment of BPV-1 DNA contains an intact human β-globin gene which is actively transcribed in the mouse fibroblasts transformed by these recombinants (DiMaio *et al.*, 1982). RNA mapping experiments using nuclease S1 and primer extension with reverse transcriptase indicate that the β-globin gene in the BPV-1 transformants is transcribed from the promoter normally active in adult red cell precursors (although this promoter is normally inactive in fibroblasts), that both intervening sequences are faithfully removed, and that a poly(A) tail is affixed at the correct site. It was estimated that between 0.1 and 1% of the mRNA in the transformed cells is globin-specific; this is at least two orders of magnitude higher than the amount of globin RNA in mouse L cells containing a transferred human β-globin gene (Charney *et al.*, 1984). Such high expression is somewhat surprising in light of several reports that the levels of BPV-1-specific RNA are exceedingly low in transformed cells (Heilman *et al.*, 1982; Amtmann and Sauer, 1982; Engel *et al.*, 1983), but numerous other cloned genes on BPV-1 vectors are expressed at a very high level. In addition to globin RNA with the correct 5′ end, a small amount of RNA initiated farther upstream is produced when the globin gene is in the same transcriptional orientation as the BPV-1 genes, but not when it is in the opposite orientation.

The generation of stable cell lines producing large amounts of globin mRNA prompted Treisman *et al.* (1983) to use this system to investigate

the transcripts produced from mutant globin genes isolated from patients with the hereditary hemoglobin disorder, beta thalassemia. Mouse cell lines were generated with BPV-1 plasmids containing these mutant genes in place of the normal one. Nucleotide substitutions within the globin gene often resulted in the appearance of incorrectly processed RNA molecules which were unable to produce normal globin polypeptides. In one case, a single base change resulted in the utilization of a normally cryptic splice site several hundred nucleotides removed from the location of the mutation. This approach of using BPV-1 vectors to generate cell lines producing large amounts of RNA for structural analysis should be widely applicable. However, it should be noted that gross rearrangement of the transferred DNA can be accompanied by the utilization of abnormal splicing and polyadenylation signals as has been reported for an integrated, rearranged BPV-1-linked dihydrofolate reductase gene (Breathnach, 1984).

B. Metallothioneins

The metallothioneins are small, cysteine-rich polypeptides which bind and detoxify heavy metal ions. The expression of the metallothionein genes is normally stimulated by heavy metals or by the glucocorticoid, dexamethasone. The concentration of RNA transcribed from the extrachromosomal mouse metallothionein I, the human metallothionein II_A, and the human metallothionein II_B promoters is increased two- to tenfold after administration of zinc or cadmium, indicating that the genes do not have to be located in a specific position in a cellular chromosome for this response (Pavlakis and Hamer, 1983a; Karin *et al.*, 1983; Richards *et al.*, 1984). When these genes are carried on a stable plasmid vector, there is remarkable uniformity in basal and induced expression levels in independently derived cell lines (Karin *et al.*, 1983).

Dexamethasone does not induce the metallothionein genes on the BPV-1 recombinants although this treatment does induce the endogenous cellular genes. The differential response of the BPV-1-linked but not the endogenous genes to metals and glucocorticoids indicates that dexamethasone does not normally induce by increasing intracellular levels of heavy metal. The same human metallothionein II_A promoter used in these BPV-1 experiments has also been stably incorporated into the chromosomal DNA of human cells, and in some of these cell lines it is induced by dexamethasone (Richards *et al.*, 1984). Therefore, the lack of induction in the BPV-1 system cannot be caused by the absence of a signal from the metallothionein gene fragment and may instead be due to the extrachromosomal state of the DNA or to the use of the heterologous NIH 3T3 host cell.

As mentioned in a previous section, it is possible to select BPV-1-transformed mouse cells containing extrachromosomal mouse and human metallothionein genes. Moreover, to facilitate the expression of other

genes that would normally not be actively transcribed, Pavlakis and Hamer have developed a set of cloning vectors where foreign DNA can be inserted in such a manner as to come under the control of the mouse metallothionein I promoter which is expressed at a high level in cells transformed by the BPV-1–metallothionein recombinants (Pavlakis and Hamer, 1983a,b). Using this sort of vector, 2–6 $\times$ 10^8 molecules/cell of growth hormone are secreted daily by some cell lines containing a BPV-1-linked human growth hormone gene fused to the metallothionein promoter. This level of expression is about tenfold higher than in cells infected with the human growth hormone gene on a lytic SV40-based vector (Hamer and Walling, 1982).

C. Hepatitis B Surface Antigen

Infection by hepatitis B virus affects hundreds of millions of individuals worldwide. A safe and effective hepatitis B vaccine is being produced from hepatitis B surface antigen (HBsAg) purified from the serum of chronic carriers, but the cost of the vaccine prohibits its widespread use in developing countries and the possibility that it contains as-yet-unrecognized infectious agents must be considered. Furthermore, the requirement for extensive posttranslational processing of the primary translation product of HBsAg mRNA makes the antigen an unlikely candidate for economic, large-scale production in bacteria, which cannot carry out these modifications. These considerations have led a number of groups to use the HBsAg gene linked to BPV-1 DNA to generate stable mouse cell lines that secrete large amounts of mature HBsAg. Although the experimental details differ, the general experience has been that most cell lines selected for morphological transformation by the HBsAg-linked BPV-1 DNA produce detectable and often very high levels of HBsAg (Stenlund *et al.*, 1983a; Wang *et al.*, 1983; Hsiung *et al.*, 1984). The glycosylated antigen is secreted into the culture medium as lipoprotein particles with buoyant density, electron microscopic appearance, polypeptide composition, and immunogenicity indistinguishable from comparable 22-nm particles purified from the serum of chronic hepatitis carriers.

To increase levels of expression, Hsiung *et al.* (1984) placed the gene under the control of regulatory signals derived from the mouse metallothionein I gene. In addition to obtaining high levels of gene expression, they found that they could extend the life of the cell cultures at confluence by an alternating regimen of serum-containing and serum-free culture media. In this manner they were able to maintain high-expressing cell lines in roller bottle culture for 60 days without passaging. Such *in vitro* manipulation of recombinants to maximize expression levels and improved cell culture techniques to increase yield may allow large-scale production of commercially important polypeptides. Although the level of gene expression achieved so far with BPV-1 vectors has probably not

TABLE III. Production of Hepatitis B Virus Surface Antigen in Mammalian Cell Cultures

Vector	Host	Amount[a]	Duration[b]	Reference
—	PLC/PRF/5 (human hepatoma cell line)	0.2–0.3	—	Alexander *et al.* (1976), Stratowa *et al.* (1982), Wang *et al.* (1983)
Viral DNA[c]	L tk⁻ (mouse)	1–2	7 days	Dubois *et al.* (1980)
Viral DNA[d]	NIH 3T3 (mouse)	8–10	30 cell passages	Christman *et al.* (1982)
Moloney murine sarcoma virus	NIH 3T3 (mouse)	4.5	1 week	Stratowa *et al.* (1982)
SV40	CV-1 (monkey)	1.25	3 days	Moriarty *et al.* (1981)
SV40	CV-1 (monkey)	38	2 weeks	Liu *et al.* (1982)
SV40	COS-1 (monkey)	45	2–3 weeks	Crowley *et al.* (1983)
SV40	COS-1 (monkey)	16–20	60 hr	Siddiqui (1983)
BPV-1	C127 (mouse)	6	1 month	Stenlund *et al.* (1983a)
BPV-1	NIH 3T3 (mouse)	6	6 months	Wang *et al.* (1983)
BPV-1[e]	C127 (mouse)	10–20	5 months	Hsiung *et al.* (1984)

[a] μg hepatitis B surface antigen/10^7 cells per day.
[b] Length of time cell cultures produced antigen.
[c] Hepatitis B virus DNA was cotransferred with herpes simplex virus thymidine kinase gene.
[d] Hepatitus B virus DNA was cotransferred with a dihydrofolate reductase gene, and expression was assayed after gene amplification in methotrexate.
[e] Surface antigen gene was transcribed from a mouse metallothionein I promoter.

been optimized, it compares favorably with the HBsAg yields reported for a variety of other host–vector systems (Table III).

D. Human β-Interferon Gene

The interferons are secreted proteins that play an important role in the defense against viral infection. Because of their therapeutic potential and because they represent a class of inducible genes, the interferon (IFN) genes have been studied using several types of gene transfer experiments. The human β (or fibroblast) IFN gene has been transferred into mouse C127 cells using two different types of BPV-1 vectors. In the earlier experiments, the IFN-β gene linked to the subgenomic transforming fragment of BPV-1 DNA was excised from pBR322 sequences prior to transformation (Zinn *et al.*, 1982; Mitrani-Rosenbaum *et al.*, 1983). Many cell lines selected for morphological transformation secreted human IFN into the culture medium. The secreted IFN activity and IFN RNA levels (but not BPV-1 RNA levels) were increased by treatment of the cells with either poly(rI · rC) or Newcastle disease virus, two well-studied inducing agents of the IFN-β genes. Induction is also caused by cycloheximide treatment (Maroteaux *et al.*, 1983). Although the DNA appeared extrachromosomal in the transformants, there was considerable DNA rear-

rangement among different cell lines. Perhaps as a consequence of these diverse genomic configurations, the expression levels and extent of induction varied widely among different cell lines established with the same DNA fragment.

The results were more satisfactory when the same gene was transferred using a stable plasmid vector containing the 5500-bp segment of BPV-1 DNA and the stimulatory segment of human globin DNA (Zinn *et al.*, 1983). With this recombinant, most transformed cell lines contained plasmid DNA identical to the input molecule. Moreover, the basal and induced level of human IFN RNA and secreted IFN activity were essentially identical in all cell lines even though the latter were not subcloned from single transformed cells (Table IV). In contrast, other methods of establishing cell lines stably expressing a transferred gene are characterized by substantial variability of expression among different cell lines. For example, several laboratories have used biochemical selection to establish cell lines containing a transferred integrated human IFN-β gene; within each set of cell lines the extent of inducibility varied at least tenfold (Canaani and Berg, 1982; Ohno and Taniguchi, 1982; Pitha *et al.*, 1982; Hauser *et al.*, 1982).

In the BPV-1 system, induction of the wild-type IFN gene with poly(rI · rC) resulted in an approximately 400-fold increase in the level of correctly initiated human IFN-β, but in only a 40-fold increase in human IFN activity. The endogenous mouse fibroblast IFN activity was induced at least 2000-fold. Low levels of expression of the transferred IFN-β gene were also seen with the unstable linearized BPV-1 vectors and with other non-BPV-1 vector systems (Pitha *et al.*, 1982; Ohno and Taniguchi, 1982; Hauser *et al.*, 1982; Canaani and Berg, 1982). The factors responsible for this suboptimal regulatory response have not been defined.

The lack of variability in gene expression among cell lines enabled

TABLE IV. Interferon Induction in Mouse Cells Transformed with pBV-IFΔ3[a,b]

Cell line	Human IFN activity (units/ml)		Mouse IFN activity (units/ml)		Induced/uninduced human IFN activity
	−	+	−	+	
3-1	200	2000	<20	40,000	10
3-5	400	4000	ND	ND	10
3-6	300	4000	<20	40,000	13
3-7	200	2000	<20	40,000	10
3-8	200	2000	<20	40,000	10
3-9	400	4000	<20	40,000	10
3-10	400	4000	ND	ND	10

[a] From Zinn *et al.* (1983) with permission.

[b] Several independent cell lines were established with pBV-IFΔ3, a plasmid containing a deletion in the 5′ flanking region of the human IFN-β gene. Secreted IFN activity was measured for each cell line in the absence of induction (−) or after treatment with poly(I·C) (+). IFN secretion by this mutant is induced about 10-fold; the wild-type gene is induced about 40-fold.

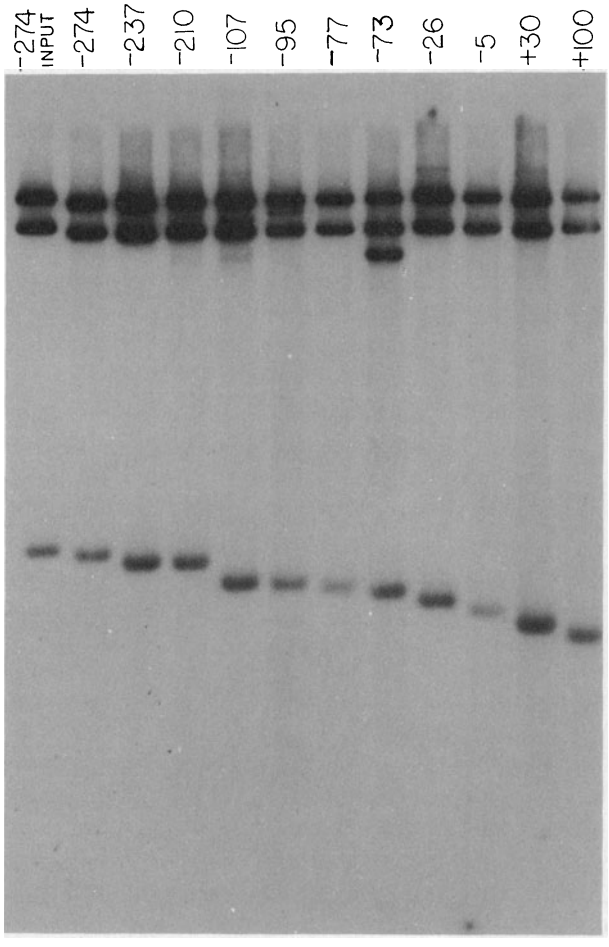

FIGURE 3. Structure of interferon gene deletion mutants in transformed mouse cells. C127 cells were transformed with a series of interferon gene deletion mutants, and individual foci were expanded into cell lines from which low-molecular-weight DNA was isolated. After digestion with restriction endonucleases, the DNA fragments were separated by agarose gel electrophoresis and the transferred DNA was detected by hybridization. The numbers above each lane indicate the mutant plasmid used for transformation, and the lowest band in each lane is the DNA fragment which contains the constructed deletion. (From Zinn *et al.*, 1983, with permission.)

Zinn *et al.* (1983) to assess the effect of a series of mutations in the putative regulatory region of the human IFN gene. Deletions in the 5′ end of the gene were constructed *in vitro* and transferred individually into cells. Several transformed cell lines were generated from each mutant and were analyzed for DNA content and for IFN gene expression. In most of the transformants, the transferred DNA had the same structure as the input molecule (Fig. 3). Analysis of IFN expression confirmed the lack of variability within each set of cell lines and indicated that there are two regulatory regions adjacent to the 5′ end of the human IFN gene. One

region, between 107 and 210 bp 5′ from the site of transcription initiation, plays a role in maintaining a low level of transcription in the absence of induction. Genes with deletions of this region also exhibit altered induction kinetics. The second region, located between 19 and 77 bp 5′ from the gene, is required for increased accumulation of IFN RNA after induction. Recently, it has been found that a 40-base segment normally located between 37 and 77 bp 5′ to the IFN gene is sufficient to confer high levels of inducibility to heterologous BPV-1-linked promoters (Goodbourn *et al.*, 1985). Similar analysis of the effects of mutations and isolated regulatory segments on other genes will probably be one of the major uses of BPV-1 vectors in the near future. BPV-1-linked human IFN-γ and IFN-α genes have been expressed in mouse cells as cDNA clones driven from the SV40 early promoter (Fukunaga *et al.*, 1984).

E. Mouse Mammary Tumor Virus

Transcription of the mouse mammary tumor virus (MMTV) is stimulated by glucocorticoids, and gene transfer experiments have localized the hormonally responsive element to the long terminal repeat of this retrovirus (reviewed in Ringold, 1983). To develop a completely defined model system to investigate this response, Ostrowski *et al.* (1983) constructed BPV-1 recombinants containing the MMTV LTR and introduced them into C127 cells after removing bacterial plasmid sequences. Between 50 and 200 copies of the transferred DNA were maintained extrachromosomally in the transformants, but in contrast to the common experience with BPV-1 vectors excised from pBR322, there was little or no evidence of rearrangement. RNA analysis indicated that there was a great deal of variability in gene expression in the absence of induction and that dexamethasone treatment of cells caused the accumulation of increased amounts of RNA initiated both at the MMTV promoter and at sites farther upstream (but caused no change in the number of DNA templates). The extent of this increase varied from 1.3- to 100-fold among different cell lines, perhaps reflecting either minor, undetected DNA rearrangements or an inherent property of the MMTV promoter. The latter possibility is suggested by the variability of transcriptional induction in cells containing MMTV proviruses (Ringold, 1983)

Nuclei were prepared from transformed cells grown in the presence or the absence of dexamethasone, and recombinant minichromosomes were extracted using a procedure that resulted in approximately a 500-fold enrichment of extrachromosomal molecules. Nascent transcripts were then elongated *in vitro* and the newly synthesized RNA was analyzed. The results of this experiment indicate that hormonal induction in this system takes place at the level of transcription initiation. Importantly, these experiments also demonstrate convincingly that induction is occurring on extrachromosomal templates.

F. Polypeptide Hormones

Genes for several polypeptide hormones have been introduced into mouse cells on BPV-1 vectors; in all cases the hormones are secreted from the transformed cells. The results with the rat preproinsulin gene (Sarver *et al.*, 1981) were discussed above (see Section III). The BPV-1-linked rat growth hormone gene is also expressed in transformed cells (Kushner *et al.*, 1982). The cell lines reported in this study are not suitable for studies of some aspects of the regulation of growth hormone gene expression because transcription of the transferred gene does not respond to dexamethasone, an inducer of the endogenous gene. Moreover, the growth hormone RNA in the transformants initiated upstream from the initiation site of bona fide rat growth hormone mRNA.

As summarized in Section IV.B, BPV-1 vectors have been used to produce high levels of human growth hormone (Pavlakis and Hamer, 1983b). Using a similar vector, Ramabhandran *et al.* (1985) reported high-level production of bovine growth hormone able to specifically bind growth hormone receptors. The gene encoding the common α subunit of the human glycoprotein hormones has also been introduced into mouse cells, and the synthesis, glycosylation, and secretion of the protein have been described (Ramabhandran *et al.*, 1984).

G. Other Genes

BPV-1 vectors have been used to introduce a number of other genes into cells. Stable mouse cell lines have been generated which express the vesicular stomatitis virus glycoprotein (a protein which mediates membrane fusion at low pH) or a mutant version lacking the COOH-terminal membrane anchor sequence (Florkiewicz *et al.*, 1983; Florkiewicz and Rose, 1984). Transcription of these genes was initiated at a linked SV40 promoter. At least 95% of the transformed cells synthesize the glycoprotein, but the level of expression varied greatly from cell to cell. Acidification of the medium caused the transformed cells to fuse, indicating that the viral protein was biologically active. Comparison of expression of the wild-type and mutant genes demonstrated that the mutant protein was secreted from the cells rather than anchored into the membrane. Moreover, the attachment of complex oligosaccharides and transit from the rough endoplasmic reticulum to the Golgi apparatus were delayed for the mutant. Similar experiments have been performed with a BPV-1-linked influenza virus hemagglutinin gene (Sambrook *et al.*, 1985). The authors suggest a novel use of cell lines expressing the viral hemagglutinin: the ability of red cell ghosts to bind these cells and to disgorge their contents may allow the efficient delivery of macromolecules into the cytoplasm of cells.

A cellular surface antigen has been expressed using BPV-1 vectors as

well. A human class I histocompatibility antigen heavy chain polypeptide is synthesized at a very high rate in C127 cells transformed with its gene linked to BPV-1 DNA (DiMaio *et al.*, 1984). The heavy chain associates with β_2-microglobulin at the cell surface of these transformants, but the protein seems less stable than the corresponding protein in human lymphoblastoid cells.

C127 cells have also been transformed with BPV-1 recombinants containing the capsid protein genes of the minute virus of mice (MVM), an autonomous parvovirus, and these cells secrete MVM capsids (Pintel *et al.*, 1984). Immunofluorescence experiments demonstrated a great deal of variability in both the amount of capsid proteins expressed and the intracellular localization of these proteins. These results were obtained even though the cells were cloned several times, and the molecular basis of this variability is obscure. A human U1 RNA gene has been introduced into mouse cells on a variety of BPV-1 vectors (Schenborn *et al.*, 1985). As much as 15% of the total U1 RNA in the transformed cells was encoded by the transferred gene, and no more than several hundred base pairs of flanking DNA sequence were required for efficient synthesis.

V. STABILITY OF BPV-1 RECOMBINANTS

The precise structure of BPV recombinants seems to play a major role in determining plasmid stability. One of the globin DNA-containing vectors, pBPV-BV1, appeared more stable than several other vectors when linked to the influenza virus hemagglutinin gene (Sambrook *et al.*, 1985), but it is premature to generalize from a single set of recombinants. When the goal of a gene transfer experiment is the establishment of permanent, stable cell lines, it is essential that the DNA is maintained without rearrangement. Although these conditions are often met with BPV-1 vectors, examples of plasmid instability are numerous. Transformation with linear BPV-1 molecules almost invariably results in circularization with rearrangement of the DNA molecule at its ends and acquisition of new DNA sequences (Law *et al.*, 1981; Sarver *et al.*, 1981; Zinn *et al.*, 1982). BPV-1 vectors that transform cells as intact plasmid molecules eliminate this problem, but with continued passage of the resulting cell lines, deletions can occur even in these plasmids (Fig. 4) (DiMaio *et al.*, 1982). These deletions often appear first in the bacterial plasmid segments of the recombinants, suggesting that these segments may be selected against during plasmid replication. If the ability to rescue the recombinant from mouse cells is not a prerequisite of an experiment, it may be advisable to remove the bacterial plasmid sequences and circularize the BPV-1/foreign gene segment *in vitro* prior to transformation. Apparent oligomerization of both unrearranged and deleted plasmids has also been reported (Binetruy *et al.*, 1982), but segregation of BPV-1 plasmids from transformed cells appears to occur very infrequently (Turek *et al.*, 1982).

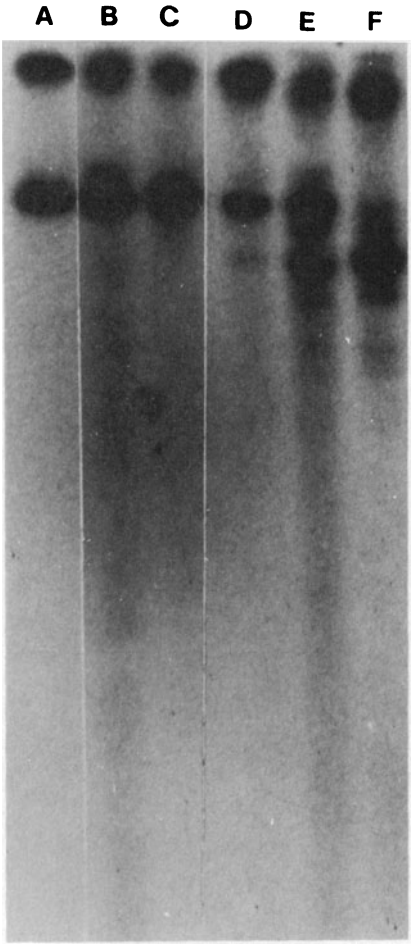

FIGURE 4. Generation of deleted plasmids during passage of BPV-1-transformed mouse cells. C127 cells were transformed with pBPV-β1 or pBPV-β3 (DiMaio *et al.*, 1982), and cell lines were cloned from single transformed cells. Low-molecular-weight DNA was isolated from these lines every five passages (15–20 cell divisions) and compared by gel electrophoresis and hybridization to a probe containing BPV-1 DNA. A: marker DNA (pBPV-β1). B, C: DNA from cell line transformed with pBPV-β1, after 5 and 10 passages. D–F: DNA from cell line transformed with pBPV-β3, after 5, 10, and 15 passages. Deleted plasmids arise in the latter cell line as the cells are passaged.

Inclusion or removal of specific DNA sequences can also result in instability. When a murine retroviral transcriptional enhancer is inserted into MMTV/BPV-1 recombinants, integration and rearrangement occur in most cell lines (Ostrowski *et al.*, 1983). When a portion of the stimulatory segment of human globin DNA is removed from a plasmid containing the 5500-bp fragment of BPV-1 DNA, the resulting molecule still transforms efficiently and is maintained as a plasmid; but the transferred DNA is frequently rearranged (Zinn *et al.*, 1983). Although most deletions in the 5′ portion of the human IFN-β gene are compatible with stable plasmid maintenance, a few deletion mutants generated exclusively cell lines that were unstable in terms of either the structure of the plasmid DNA or IFN gene expression (Zinn *et al.*, 1983). The position of the human DNA insert influenced the stability of BPV plasmids containing the human U1 RNA gene (Schenborn *et al.*, 1985). DNA segments could cause instability by inhibiting the production of virus-encoded *trans*-acting factors required for plasmid replication or segregation or by interfering with

viral sequences that are required in *cis* for these phenomena. Because only the *cis*-acting sequences need be physically joined to the foreign DNA to ensure its extrachromosomal propagation, perhaps it will be possible to supply *trans*-acting replication factors from a second viral genome in the cell, thus eliminating the possibility that a linked foreign DNA segment will adversely affect the production of proteins required for replication. Recombination between multiple plasmids in a single cell and segregation of a plasmid which is not maintained by selection are potential pitfalls of this approach, but Lusky and Botchan (1984) have reported the stable co-maintenance of a wild-type BPV-1 genome supplying *trans* replication factors and a plasmid containing only a small segment of BPV-1 DNA, which presumably supplies the *cis* replication requirements for the plasmid. Although it has been reported that the absence of adenine methylation stabilized a BPV recombinant containing a thymidine kinase gene (Lusky *et al.*, 1983), this does not seem to be a general effect (Schenborn *et al.*, 1985).

Because viral DNA is not encapsidated in the BPV-1 system, there is no obvious size limitation on BPV-1 recombinants. Stable 16,000-bp recombinants have been reported (DiMaio *et al.*, 1982). Transfer of a 24,000-bp plasmid resulted in its conversion into a higher-molecular-weight (possibly integrated) form; it has not been determined whether this behavior was a consequence of its size or some other feature or the plasmid (DiMaio *et al.*, 1984).

As is clear from this brief discussion, there are probably numerous factors that influence plasmid stability, and they are still poorly defined. All cell lines generated with BPV-1 recombinants must be evaluated separately for the presence of DNA rearrangements and integration. The presence of a moderate amount of rearrangement may not be a serious problem for some experiments such as the production of high levels of gene products (e.g., Treisman *et al.*, 1983; Stenlund *et al.*, 1983a). Moreover, plasmids can be rescued by transforming bacteria with DNA from mouse cell lines containing predominantly integrated or oligomerized BPV-1 recombinants (Sekiguchi *et al.*, 1983; Binetruy *et al.*, 1982). As more experience is gained in the use of these vectors and as the molecular biology of the papillomaviruses is studied, perhaps the rules governing plasmid stability will emerge.

VI. FUTURE DIRECTIONS

Some of the numerous potential applications of BPV-1 vectors have already been discussed. Their use in generating cell lines synthesizing large amounts of RNA or mature gene products has been amply demonstrated. To synthesize products that are normally toxic to the cells, it may be possible to construct vectors which allow the plasmid copy number or transcription to be acutely increased after cell lines have been established. Attempts to use the mouse metallothionein I promoter in this manner have met with limited success. Only a modest level of in-

duction has been observed for the human growth hormone structural gene fused to this promoter (Pavlakis and Hamer, 1983a); no induction was seen for an HBsAg gene fused to the same promoter (Hsiung *et al.*, 1984). Gene fusions using the MMTV or the human IFN-β promoter may be more effective.

As is shown by the mutational analysis of the human IFN-β gene (Zinn *et al.*, 1983), the uniform levels of expression of BPV-linked genes make this an ideal system for quantitative evaluation of regulatory mutants. Goodbourn (personal communication) has recently found similar levels of expression in cell lines derived from individual foci and in pooled cell lines descended by relatively few cell divisions from multiple, independent, transformed cells. Use of pooled cell lines should greatly speed the analysis of mutants; the short period of cell expansion may also allow the analysis of recombinants which are not stable enough to tolerate the extended cell culture required to generate a cell line from a single cell. Unfortunately, many regulatory phenomena cannot be studied in mouse fibroblasts. The use of biochemical selection may allow the transfer of BPV-1-linked genes into a wide range of cells, but it has not been determined whether BPV-1 DNA will replicate as a plasmid in many cell types that are resistant to morphological transformation by the virus. It is unlikely that uniform expression levels will be achieved unless the BPV-1 vector is maintained extrachromosomally.

The inability to purify specific segments of chromatin from the bulk of cellular chromatin has necessitated the use of indirect, hybridization techniques to analyze the chromatin associated with specific cellular genes. Using BPV-1 vectors, it is now possible to isolate and partially purify chromatin associated with specific genes. Such preparations can be examined biochemically or with the electron microscope, as has been done for BPV-1 chromatin (Rösl *et al.*, 1983). The relatively low copy number of BPV-1 plasmids in cells will make biochemical characterization a formidable task, but it also makes it less likely that important regulatory molecules will be titrated out by massively replicating templates, as may be the case with some other viral vectors.

There is also a great deal of interest in exploiting BPV-1 recombinants to isolate plasmids that have undergone sequence alterations during replication in mouse cells. For example, after growth of a mouse cell line containing a BPV-1 recombinant, bacteria can be transformed with plasmids purified from these cells; if the appropriate selection schemes can be devised, it should be possible to isolate altered plasmids which have undergone mutation or recombination. Plasmids that confer a selectable phenotype on the transformed mammalian cells can also be isolated (Fig. 5). In one straightforward sort of experiment, a gene cloned on BPV-1 is mutagenized *in vitro* and the pool of mutagenized molecules is transferred into mammalian cells. Selection is then imposed to isolate rare transformants that have acquired a mutant gene and therefore express a selectable phenotype. Plasmid DNA is then extracted from these transformants and used to transform bacteria to amplify the variant DNA for

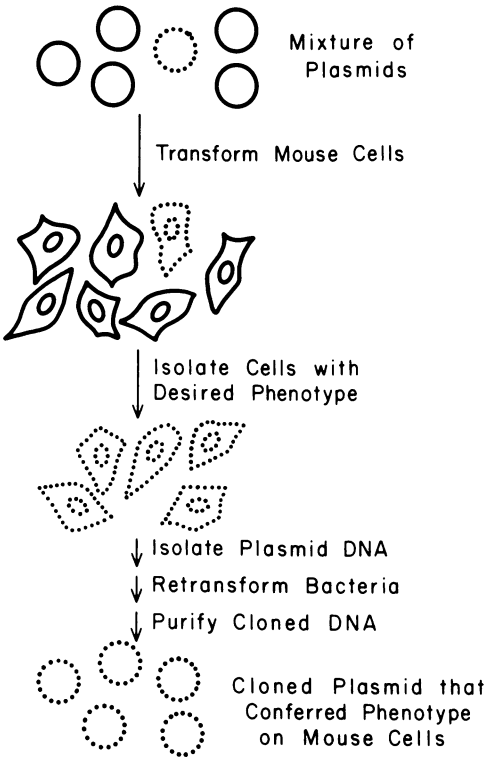

FIGURE 5. Schematic diagram of a method to rescue genes that confer a selectable phenotype on mouse cells. The circles at the top and bottom of the figure represent plasmid DNA molecules that have been amplified in bacteria. The other forms represent transformed mouse cells. Plasmids that can confer a selectable phenotype after transfer into mouse cells are represented by dotted circles; dotted forms represent cells that contain this plasmid. If the plasmid mixture initially used to transform mouse cells contains rare members that confer a selectable phenotype on mouse cells, by selecting cells with this phenotype it may be possible to isolate the responsible plasmids. (From DiMaio, 1984.)

detailed analysis. Using similar approaches, it may be possible to isolate specific genes from genomic or cDNA libraries constructed in BPV-1 vectors.

Analogous "shuttle vectors" are being developed from cosmids (Lund *et al.*, 1982; Lau and Kan, 1983), bacteriophage (Hamada *et al.*, 1983; Lindenmaier *et al.*, 1982), SV40 (Breitman *et al.*, 1982; Conrad *et al.*, 1982), retroviruses (Cepko *et al.*, 1984), and systems that require *in vitro* enzymatic manipulation (Howard *et al.*, 1983), but none offer the uniform expression levels and the ease of plasmid recovery that are potentially attainable with the BPV-1 system. Implementation of this approach obviously requires efficient selection or screening procedures that are compatible with stable extrachromosomal replication of the recombinant and with the expansion of the transformed foci into mass cultures for DNA extraction. There are numerous technical problems which must first be

overcome. The conditions used to initially transform mouse cells must generate a large number of independent transformants in order to make recovery of a rare mutant or gene feasible. Some inserts may interfere with focus formation or plasmid stability. Moreover, it will probably be difficult to construct a representative library in plasmid vectors given the problem of generating many thousands of different circular DNA molecules each containing both a BPV-1 and an inserted DNA segment. The inclusion of a bacteriophage lambda *cos* site on a BPV-1 vector may facilitate construction of such a library (Matthias *et al.*, 1983), but it has not been established that a 50,000-bp BPV-1 recombinant can be stably maintained in mammalian cells or be efficiently recovered from them.

The persistence of multiple different plasmids within a clone of transformed mouse cells poses additional problems. When an equimolar mixture of two very similar but distinguishable BPV-1 plasmids is used to transform cells, both persist in cell lines derived from individual transformed foci (DiMaio, unpublished results). This indicates that selection conditions should be designed to enable isolation of the gene of interest if it coexists in cells with other plasmids. Multiple different plasmids will be recovered in bacteria if there are multiple species in the selected mouse cells, and these recovered plasmids will then have to be rescreened individually for activity in mammalian cells. Further experiments are needed to determine whether these potential problems represent an insurmountable obstacle or merely a technical challenge.

Finally, it is clear that better papillomavirus cloning vectors will be constructed as the molecular biology of these viruses becomes better understood. Factors that influence transformation efficiency, stability, and copy number have to be identified and evaluated before vectors can be designed on a rational basis. A systematic assessment of vectors derived from a number of papillomaviruses may identify a specific papillomavirus–host cell combination that is particulary compatible with stable plasmid maintenance. Conversely, studies using papillomavirus vectors will undoubtedly play an essential role in elucidating the molecular biology of these viruses.

ACKNOWLEDGMENTS. The author wishes to thank the many colleagues who contributed material for inclusion in this chapter. In addition, the support of the Edward J. Mallinckrodt Foundation is gratefully acknowledged.

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