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Review

Use of Vaccinia Virus to Express Biopharmaceutical Products

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Recent technological advancements have fostered the continued development of vaccinia virus as an efficient eukaryotic cloning and expression vector system. Genetically engineered vaccinia virus strains have been constructed for use (i) as recombinant vaccines for the prophylaxis of infectious disease, (ii) in producing significant quantities of biologically active polypeptide factors or enzymes, and (iii) as basic research tools with which to investigate primary structure–function relationships between proteins and their catalytic activities. This review examines the basic vaccinia vector system, its advantages and limitations, and current areas of research. As a specific example of the power and utility of this approach, attention is focused on the application of this technology to the field of neurobiology, specifically the use of recombinant vaccinia to study the expression, processing, and transport of cellular neuropeptides.

KEY WORDS: vaccinia virus; expression vector; neuropeptides; protein processing.

INTRODUCTION

One of the central themes of modern molecular biological experimentation is the use of cloning and expression vectors. Using such systems, individual genes can be excised from their normal cellular environment and mobilized into heterologous cell types in order either to elucidate the structure and function of the encoded polypeptides or to facilitate the mass production of individual proteins of interest. A variety of vector systems has been developed for these purposes including bacteriophage (1,2), bacterial plasmids (3,4), yeast (5), mammalian expression plasmids (6), and a number of different animal viruses (7–11) including, most notably, vaccinia virus (12).

When compared to other expression vector systems, vaccinia offers some decided advantages. For instance, plasmid expression vectors can be used only with certain cell types that are amenable to microinjection or transfection techniques, and even then, only a small portion of the cells will take up, maintain, and express the gene of interest (13). In contrast, since vaccinia is a virus, one can synchronously infect (and hence express an inserted gene) in virtually 100% of a cell population. Furthermore, because it is an animal virus which replicates in eukaryotic cells, it can be expected that eukaryotic gene products will be correctly processed and transported, which is usually not the case using bacterial-based vectors. Other animal viruses such as retroviruses (7), papovaviruses (8), herpesviruses (9), adenoviruses (10), and baculoviruses (11) have also been used as vectors. Unfortunately, all of these virus groups replicate within the nuclear compartment of infected cells using the host cells' enzymatic machinery. This necessitates that foreign genes be placed in the proper context with respect to promoters, enhancers, splice junctions, polyadenylation sites, and RNA transport signals. Since our understanding of the nature and spatial constraints of eukaryotic regulatory signals is still rudimentary at best, the construction of a highly active chimeric gene remains a challenging proposition. Using the vaccinia system, many of these considerations are circumvented.

Vaccinia has a number of biological attributes that make it uniquely suited for vector work. (i) Unlike other DNA-containing animal viruses, vaccinia replicates in the cytoplasm of infected cells (14). This facilitates insertion of foreign genetic information into the viral genome and allows easy detection of virally encoded gene products, and since the virus does not interact with the host chromosomes, it is not tumorogenic. (ii) The broad host range of vaccinia allows genetic information to be shuttled between a variety of species and cell types. Vaccinia can be used to express foreign genes in cells from organisms that span the evolutionary tree from man to mouse to insect (15). (iii) The vaccinia virion and genomic DNA molecule are quite large, which accommodates large and/or multiple foreign inserts (16). Thus, unlike other viruses whose capacity for foreign inserts is determined by capsid size, there does not appear to be any headfull problem using vaccinia. (iv) Transcription and processing of vaccinia virus genes are carried out in the cytoplasm of infected cells by viral enzymes under the direction of viral regulatory signals, thus obviating the necessity of foreign inserts containing splicing or transport signals. All of the evidence gathered thus far indicates that if the coding sequences of a foreign gene are placed downstream of a vaccinia promoter, they will be transcribed and expressed at a high level. (v) Vaccinia is a vaccine strain to begin with, making it safe to work with in the laboratory as well as an

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appropriate background for the development of recombinant vaccine strategies.

THE BASIC METHOD

Using the marker rescue/DNA transfecton techniques that have been so elegantly developed in the laboratories of Moss (17) and Paoletti (18), the insertion of a foreign DNA into vaccinia virus (VV) and the subsequent isolation of recombinant viruses have become a relatively straightforward procedure. The rudimentary elements of this procedure are diagrammed in Fig. 1, which depicts how one might go about constructing a recombinant vaccinia strain that could be used as a vaccine against a pathogenic viral disease agent. Additional molecular details can be obtained by consulting Rice *et al.* (19).

Cloning

Genetic material from virtually any source can be cloned into and expressed by VV. The only known limitation is that the genetic information must be noninterrupted (i.e., not contain any intervening sequences), as VV does not use splicing reactions as part of its mRNA maturation pathway.

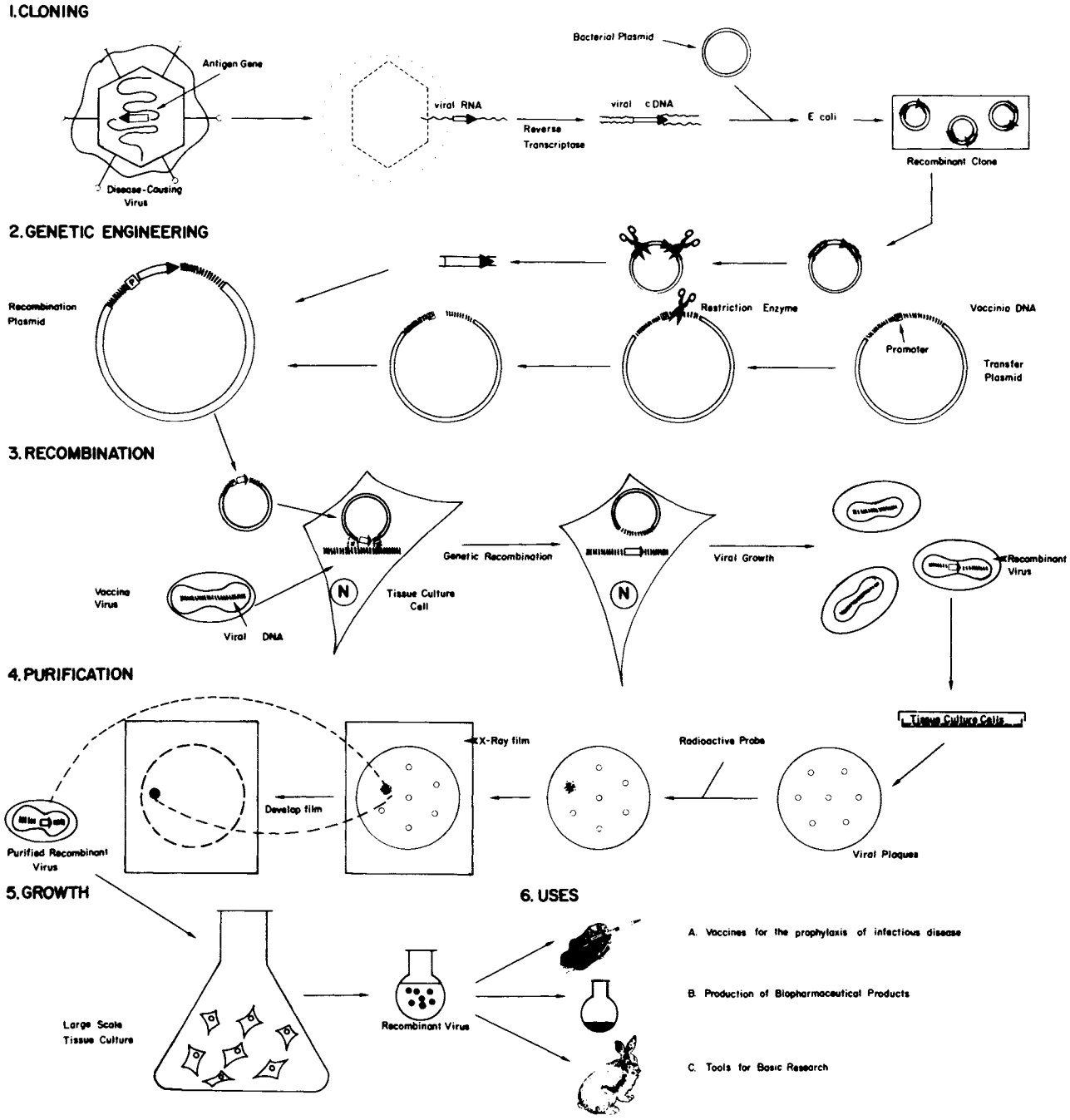

Fig. 1. Construction of recombinant vaccinia virus. At the top are outlined the essential genetic engineering and tissue culture manipulations required to construct and isolate a recombinant vaccina virus (VV).

In the example (Fig. 1), a pathogenic RNA virus is grown up and purified, and its genomic RNA extracted. This RNA serves as a suitable substrate for cDNA cloning procedures, and a cDNA library is created in *Escherichia coli* or bacteriophage. The clone encoding the protein (antigen) of interest is located by either hybridization or immunological screening procedures, and the nucleotide sequence of the cDNA insert determined. In this manner, a clone containing the entire coding sequence of the antigen can be either located or pieced together from several partial clones. Having the nucleotide sequence available then allows determination of the appropriate restriction endonuclease cleavage sites to enable the genes to be excised from the plasmid in which it resides.

Genetic Engineering

The DNA sequences encoding the gene of interest are excised and cloned directionally into a transfer plasmid. These transfer plasmids have several important features. First, they contain a bacterial plasmid backbone which includes an origin of replication and an antibiotic resistance locus to allow the plasmid to be grown and amplified in *E. coli*. Second, they contain a fragment of VV DNA sufficient in size and sequence to target foreign inserts to nonessential locations within the VV genome. Most commonly this DNA fragment is the portion of the VV genome (*Hind*III J DNA fragment) which encodes the VV thymidine kinase (*tk*) gene because this gene is known to be nonessential in tissue culture and potential VV recombinants can be selected on the basis of their tk^- phenotype acquired by virtue of insertional inactivation of this gene as a consequence of genetic recombination. Finally, in the middle of the VV sequences an efficient VV promoter element, usually abutted to a polylinker region, has been inserted. A variety of VV promoters has been used in this way but the most typical construction contains the VV 7.5-kd gene promoter. The use of this promoter is particularly advantageous since it is highly expressed at both early and late times postinfection. Thus, this provides a situation in which foreign genes being driven by this promoter are essentially turned on constitutively during infection. Once assembled, the chimeric gene cassette should be imbedded within VV DNA sequences in a transcriptionally active configuration that is sufficient to catalyze the introduction of the foreign sequences into the VV genome by homologous recombination, hence it is referred to as a recombination plasmid at this point.

Recombination

The recombination plasmid which has been coprecipitated with carrier DNA is introduced into the cytoplasm of susceptible host cells, such as L cells, by calcium phosphate-mediated transfection procedures. After a short recovery period the cells are superinfected with wild-type VV. During the process of viral DNA synthesis, which occurs at 2 to 4 hr postinfection within the cytoplasmic compartment of infected cells, the VV DNA sequences that are present within the recombination plasmid are presumably able to interact with their sister sequences in the replicating VV DNA and catalyze homologous recombination. This results in the insertion of the foreign gene into the viral genome by a double reciprocal crossover event. The molecular details of this reaction and the enzymes that catalyze it are unknown at present. As the viral replicative cycle progresses, the recombinant DNA molecule will be replicated and packaged up into progeny virions. If the foreign gene has not inactivated an essential viral gene, such recombinants should be viable and able to be recovered. The process of recombinational insertion of foreign genes is remarkably efficient. For a moderate-sized insert (<1000 bp) the fraction of progeny virions that are recombinants will range somewhere between 0.01 and 2%, even in the absence of selection. If enrichment techniques such as the use of BUdR to select for the tk^- phenotype or cotransfection of a second selectable marker are employed, this fraction can become considerably higher.

Purification

The mixture of progeny virions, containing both recombinant and wild-type VV, are plated onto monolayers of tissue culture cells at an appropriate dilution to give 100 to 1000 viral plaques per dish. After allowing the plaques to develop, they are visualized by staining the monolayers with a vital dye such as neutral red. The entire monolayer is lifted onto a nitrocellulose disk. This disk is replicated onto a reference disk which is frozen to preserve the viability of the virus contained in the plaques. The initial filter is then subjected to hybridization procedures using a radioactive probe which is specific for the foreign sequences. The plaques derived from recombinant VV are located by autoradiography, and the corresponding portion of the duplicate filter is excised with a sterile paper punch. Infectious virus is recovered from the filter by mild sonication. To ensure purity of the isolate, the recombinant viruses are then subjected to two additional rounds of plaque purification. At this point a small amount of recombinant virus is grown up, its DNA is extracted and analyzed by digestion with restriction endonucleases and Southern blot hybridization to ensure that the recombinant genome contains the gene of interest in the proper genomic context.

Growth

Once the genomic authenticity of the recombinant virus is established it is ready for larger-scale growth and purification. Suspensions of Hela or L cells are infected with VV at a low multiplicity for 48–72 hr, after which time the virus is purified from the infected cells. Routinely, one can anticipate on the order of 5–8 mg of purified virus per liter of infected cells, with a titer of about 1×10^{10} plaque-forming units per ml. The purified virus is very stable and can be stored for years at −70°C. Depending on the nature of the inserted foreign gene, the recombinant virus is now ready for the uses for which it was designed.

Uses

Recombinant VV strains have been constructed for a number of purposes including the following.

(a) Vaccines. The methodology outlined above has been used initially to isolate a considerable number of hybrid VV vaccine strains including those that express influenza hemagglutinin (20), hepatitis B surface antigen (21), the malaria circumsporozoite antigen (22), and many others (Table I).

Table I. Summary of Immunogenic Antigens Expressed by Recombinant VV Vaccine Strains

Influenza virus hemagglutinin (20)
Hepatitis B virus surface antigen (21)
Plasmodium knowlesi circumsporozoite antigen (22)
Herpes (type 1) virus gD glycoprotein (23)
Sindbis virus E1, E2, and capsid proteins (24)
Respiratory syncytial virus G protein (25)
Herpes gD/hepatitis B influenza HA (26)
Vesicular stomatitis virus G protein (27)
Human T-lymphotrophic virus (HTLV-III) *env* protein (28)
Lymphadenopathy virus (LAV) *env* protein (29)
Epstein–Barr virus gp340 membrane antigen (30)
Friend leukemia virus *env* protein (31)
Respiratory syncytial virus F protein (32)
Murine H-2K^d histocompatibility antigen (33)

Virtually all of these live vaccine strains have already been subjected to animal trials and shown to be very effective in preventing subsequent infection by the pathogen. Such vaccines have the potential of proving useful in the prevention of a variety of human and animal diseases, but such applications await further research into viral pathogenesis and possible vaccination side effects. At the present time clinical and/or field trials of the efficacy of such recombinant viruses are prohibited by the federal regulatory agencies. It is not known when this situation may change.

(b) Biopharmaceutical Products. Since the foreign proteins encoded by recombinant VV are expressed in a biologically active form, this is an appropriate system to produce large quantities of peptides for use as immunogens or to examine their biological properties. A specific example of this type of recombinant VV, namely, one expressing a human neuropeptide, is discussed in detail below (34).

(c) Basic Research. All the evidence obtained to date indicates that foreign proteins expressed by recombinant VV are modified and transported in a manner analogous to the native situation. As an example, the Sindbis viral structural proteins are correctly cleaved, glycosylated, and transported when expressed by a VV recombinant (19). This provides an appropriate system for directed genetic manipulations to dissect these protein maturation pathways *in vivo*.

AN EXAMPLE: USE OF VV TO EXPRESS NEUROPEPTIDES

Neuropeptides are polypeptide effector molecules which are synthesized as high molecular weight precursors that are processed differentially, depending on (i) the cell type in which they are translated, and (ii) the nature of the biological stimulus received by that cell, to yield a variety of biologically active peptides (35). One of the major areas of interest in this field involves the identification of the processing pathways used by these proteins and how the cell regulates the pathway to produce the desired end product. Although cDNA clones have been available for each of the three major neuropeptides [proopiomelanocortin (POMC), preproenkephalin (PE_A), and preprodynorphin (PE_B)] for some time, progress on this problem has been slow. This is due to a number of reasons including the fact that the neuropeptides precursors are present within neural cells at vanishingly low concentrations and are rapidly processed. This has made isolation and characterization of these proteins difficult and, in most cases, precluded the production of essential immunological reagents. In an effort to overcome these problems, neuropeptide cDNAs have been inserted into a variety of eukaryotic and prokaryotic vectors. Thus far, this approach has met with minimal success. Due to the advantages discussed above, it was therefore of interest to determine if the VV vector system as outlined might provide an alternative avenue to dissecting this complex problem.

Toward that end, an intact cDNA encoding human preproenkephalin (hPE) was inserted into the *Hind*III J region of wild-type VV using the basic strategy outlined in Fig. 1 (36). The recombinant VV:hPE virus was molecularly characterized and found to have the correct genome structure and to transcribe actively the inserted hPE sequences into polyadenylated messenger RNA (Fig. 2). Although no antisera exist that are capable of recognizing the hPE precursor, there is an antiserum that will react with one of the processed end-products, met-enkephalin. Thus, protein extracts suspected to contain hPE can be assayed by digesting with trypsin and carboxypeptidase to release internal peptides and a radioimmune assay (RIA) carried out to detect and quantitate the presence of met-enkephalin sequences. VV:hPE virus was used to infect a variety of fibroblast, macrophage, and neural cell lines. In each case, a considerable amount of immunoreactive material was apparent both in the extracts from VV:hPE-infected cells and in the surrounding media. No immunoreactive material was detected in similar samples from wild-type VV-infected or control cells. If the extracts from VV:hPE-infected cells were fractionated according to size by high-pressure liquid chromatography (HPLC) prior to being subjected to the RIA, an interesting picture emerged. In BSC-40 cells, a monkey kidney cell line which should not contain an endogenous secretory pathway, most of the immunoreactive material eluted at a position expected of proenkephalin (31 kd) lacking its signal sequence. In sharp contrast, within VV:hPE-infected AtT-20 cells, a rat neural cell line that has previously been shown to be fully competent to process and secrete POMC, a variety of discrete peptide peaks was evident, whereas in the media only fully processed mature met-enkephalin was present. To confirm the identity of the immunoreactive material being detected by the met-enkephalin RIA, a HPLC purification scheme was devised for the 31-kd protein that was produced in BSC-40 cells. When the N-terminal amino acid sequence of this protein was determined it was found to match exactly the amino acid sequence precicted from the nucleotide sequence of the preproenkephalin cDNA, with the putative 24 amino acid signal sequence removed. This series of experiments is summarized in Fig. 2 (for additional details, consult Ref. 34). Similar results have been obtained using a VV:POMC recombinant (B. Thorne *et al.*, in preparation) so it would appear that VV will have wide applicability for the expression of the different neuropeptides.

These results are exciting because they open up several new avenues of research in the field of neurobiology. First, VV:hPE-infected BSC-40 cells produce a considerable amount of hPE. It can be directly visualized using autoradiography of a one-dimensional gel analysis of [^{35}S]methionine-labeled extracts from VV:hPE-infected cells. Approximately 1 μg of hPE per 10^6 infected cells can be purified by

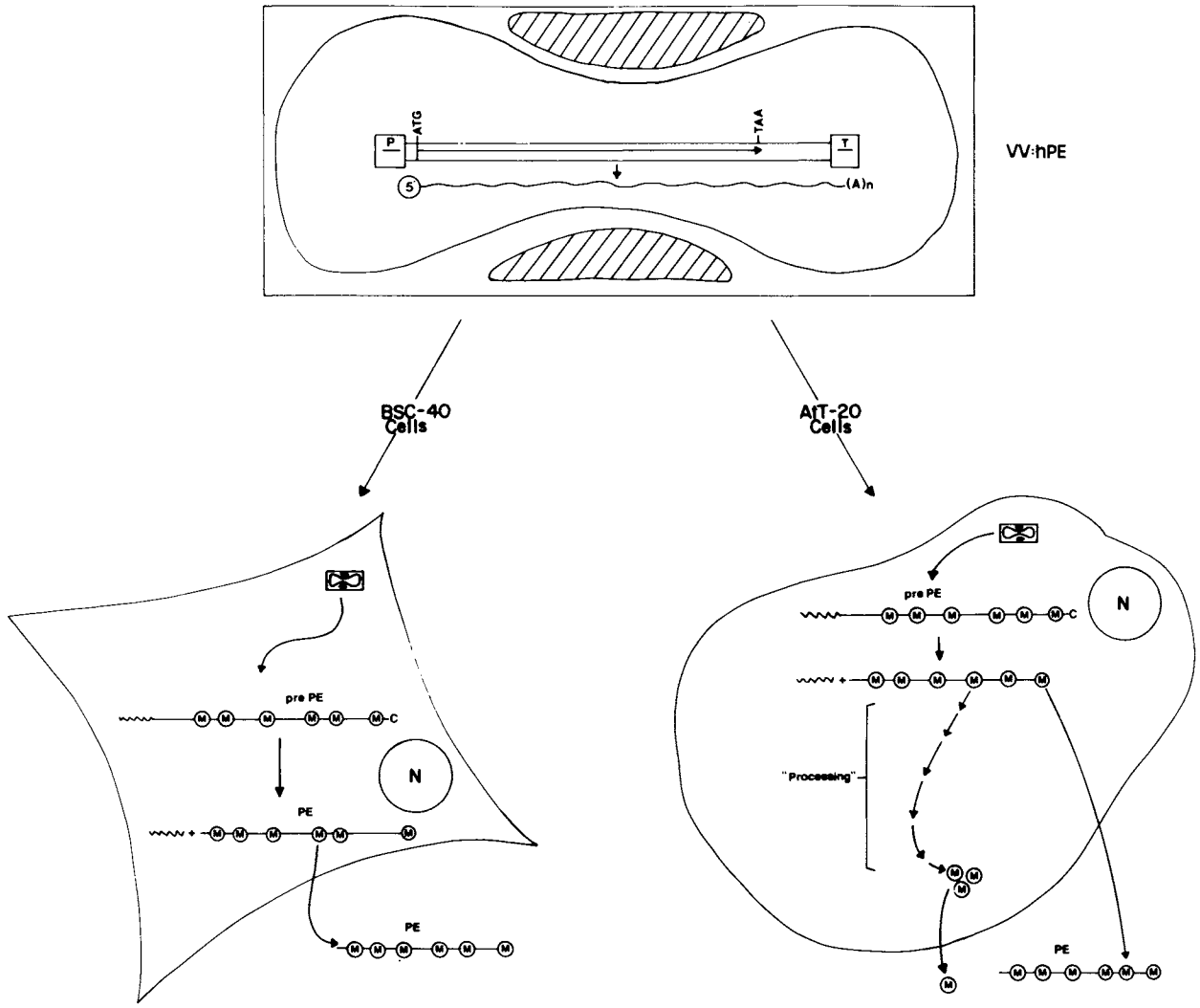

Fig. 2. Vaccinia virus/neuropeptide expression. A recombinant vaccinia virus (VV:hPE) containing an intact cDNA encoding human preproenkephalin was constructed and isolated. The structure of the chimeric gene cassette contained within this recombinant is illustrated. This recombinant was used to infect either BSC_{40} (monkey kidney) or AtT-20 (rat neural) cells in culture. The extent and nature of processing reactions of the met-enkephalin-containing (M) peptides, as determined by radioimmune assay, are indicated.

HPLC. Thus, from a liter of infected cells one could anticipate recovering approximately 0.5 mg of purified hPE with which to begin production of appropriate immunological reagents. Second, since VV:hPE produces high levels of the precursor hPE protein which is stable in BSC-40 cells, this would seem to be an appropriate assay system with which to begin identification of the cellular proteases that are normally involved in the processing pathway. The basic approach will be to insert cDNAs encoding putative protease genes into recombinant VV and then to carry out dual infections with VV:hPE to see if some or all of the processing pathway can be reconstituted in the doubly infected cells. If this type of approach is successful, then it can be carried one step further. Dual VV recombinants containing neuropeptide and protease genes could be used in concert with other VV recombinants to identify the cellular gene products that serve to regulate this pathway *in vivo* and the manner in which these factors respond to environmental stimuli. Third, it is likely that the different peptides detected in VV:hPE-infected AtT-20 cells represent processing intermediates. It therefore should be possible to purify each of these and determine the N-terminal amino acid sequences, as well as doing pulse-chase experiments in order to determine the pathway of *in vivo* processing. Finally, the recombination plasmid containing the hPE insert is an appropriate substrate for directed genetic manipulations. One should be able to remove potential modification signals as well as directing other changes in the protein structure in order to ascertain what effects these alterations have on the pattern of neuropeptide processing and/or intracellular transport and secretion. Thus we are highly optimistic that the VV vector system has been and will continue to be a very important tool in dissecting a number of important questions in the field of neurobiology.

FUTURE DIRECTIONS

It is now possible to construct routinely recombinant VV that contain and express virtually any foreign antigen or protein for which a contiguous DNA exists. This is not,

however, a trivial task, requiring numerous time-consuming labor-intensive manipulations. While sufficient for constructing effective vaccines, the current methodology is inadequate to foster the continued development of VV as generalized eukaryotic cloning and expression vector. The main limitations of the present technology are as follows. (i) Either no selection or, at best, a negative selection exists for the recombinants of interest. Thus, one is left with the situation of looking for the proverbial "needle in a haystack." (ii) Once the recombinant has been constructed, it is not possible to alter the sequence of the foreign gene *in situ*. Each individual sequence-specific mutant requires doing the mutagenesis in bacteria and then going through the entire process outlined in Fig. 1. Hence, this makes genetic engineering of the insert difficult. (iii) Vaccinia is a live virus which ultimately kills the recipient cell. This obviously limits the types and duration of experiments which can be conducted.

Research is currently ongoing in a number of poxvirus research laboratories which promises to address many of these shortcomings. Alternative coexpression transfer plasmids have been designed that contain either easily detectable marker genes such as β-galactosidase (37) or dominant selectable markers such as neomycin phosphotransferase (38), linked to a second expression cassette containing the foreign gene. This greatly facilitates the identification and isolation of recombinants of interest. Similarly, single-stranded M13 bacteriophage transfer vector systems have been developed (39) that permit site-directed mutagenesis and recombination into VV to be carried out in one step. This will permit directed genetic manipulations of VV-expressed foreign genes to be more easily conducted. Finally, efforts are being directed toward identifying ways that VV vectors can be used to express foreign genes in cells without causing cell death. Approaches under investigation in this regard include inactivation of essential VV genes that reduce viral pathogenicity (40), insertion of foreign genes into VV conditional-lethal mutants (41), and use of nonpermissive host-cell systems (15). It is anticipated that all of these efforts will greatly facilitate the use of VV as a cloning and expression vector and provide a powerful and flexible system with which to study the structure and function of eukaryotic genes.

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