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Bovine Papilloma Virus Deoxyribonucleic Acid: a Novel Eucaryotic Cloning Vector

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A novel eucaryotic vector derived from the transforming region of bovine papilloma virus was established and demonstrated to be highly effective for introducing foreign genes into animal cells. The foreign deoxyribonucleic acid (DNA) is replicated and actively transcribed as an episome, and the transcripts are translated into an authentic gene product. We have constructed a DNA hybrid molecule, BPV_{69T}-rI_1, containing the transforming region of bovine papilloma virus DNA and the rat preproinsulin gene I (rI_1), and used it to transform susceptible mouse cells. DNA hybridization analysis has demonstrated the presence of multiple unintegrated copies of hybrid DNA molecules, with the bovine papilloma virus 1 DNA segment and the rI_1 gene covalently linked in selected transformed cell lines. S1 nuclease analysis revealed the presence of a correctly spliced coding segment of the preproinsulin transcript similar or identical in its electrophoretic mobility to that of messenger ribonucleic acid produced in rat insulinoma cells. Significant levels of a protein immunoreactive with anti-insulin serum were detected by radioimmunoassay in the culture medium of transformed cells. Immunoprecipitation analysis in conjunction with competitive binding to bovine proinsulin established the identity of the protein as that of rat proinsulin.

Several methods are currently in use for delivering defined foreign deoxyribonucleic acid (DNA) segments into eucaryotic cells. These include physical injection of DNA (25), fusion of DNA-containing liposomes (6, 27) or erythrocytes (28) with target cells, and the direct application of naked DNA onto cells in the presence of calcium phosphate (7) and marker DNA (23, 36, 37).

More recently, insertion of DNA into recipient cells was achieved by using viral particles (Simian virus 40 [SV40]) in which a segment of the viral genome is covalently linked to defined nucleic acid segments (9, 10, 26). Whereas the SV40 vector system offers a rapid and efficient way to introduce foreign DNAs into permissive host cells, the system is limited by the size of DNA that can be accommodated within the virus particle. Moreover, since monkey cells are permissive for SV40 replication, infection by recombinant SV40 particles culminates in cell death. SV40 DNA has not yet been exploited as a cloning vector in nonpermissive rodent cells, because (i) SV40 transformation is associated with integration of the viral genome, a process that may disrupt the integrity of the foreign DNA segment of interest and (ii) there is no indication that the gene will be active at detectable levels from the low integrated copy numbers which are sufficient for the expression of the SV40-transforming gene.

We have been studying the biology of the bovine papilloma viruses and, in particular, their ability to transform mouse cells. In the course of these studies, several observations were made which suggested the potential usefulness of bovine papilloma virus DNA as a vector for introducing foreign genes into cells, as follows. (i) The molecularly cloned bovine papilloma virus 1 DNA as well as a cloned 69% subgenomic fragment of the bovine papilloma virus 1 genome are very efficient in inducing transformed foci in susceptible mouse cells (12, 20). (ii) Bovine papilloma virus-transformed cells contain multiple copies (10 to 120 per cell) of the viral DNA. These copies exist exclusively as unintegrated extrachromosomal molecules (M.-F. Law, D. R. Lowy, I. Dvoretzky, and P. M. Howley, Proc. Natl. Acad. Sci. U.S.A., in press), thus offering a natural means of amplifying foreign DNA sequences which are covalently linked to the bovine papilloma virus-transforming segment. (iii) Since integration of the viral genome does not occur (Law et al., in press), the physical contiguity of the "passenger" DNA segment should be preserved. (iv) The transformed phenotype

provides a marker for selecting those cells that have incorporated the foreign DNA segment; thus, any cell line susceptible to bovine papilloma virus transformation is a potential recipient. (v) Bovine papilloma virus-transformed cells grow faster than their nontransformed counterparts. This should facilitate the large-scale production of cells containing the exogenous gene and possibly, therefore, the gene product.

In this communication we report the construction of a recombinant bovine papilloma virus DNA containing the rat preproinsulin gene I and show it to be highly effective in introducing the foreign gene into mouse cells. The foreign DNA is replicated and transcribed, and the transcripts are translated into an authentic gene product.

MATERIALS AND METHODS

Cells and DNA transformation. Mouse C127 I cells (21) were maintained in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with penicillin (10 U/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal bovine serum (M.A. Bioproducts).

DNA transformation was performed by using the calcium precipitation method (7) followed by dimethyl sulfoxide enhancement (32). Briefly, a 2× DNA-$CaCl_2$ solution [1 mM tris(hydroxymethyl)aminomethane(Tris) (pH 7.9), 0.1 mM ethylenediaminetetraacetate (EDTA), 250 mM $CaCl_2$, 25 μg of calf thymus DNA per ml, 2.5 μg of recombinant DNA per ml] was added to an equal volume of 2× HBS solution (1× HBS = 140 mM NaCl, 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] 0.75 mM sodium phosphate, pH 7.1), and the DNA precipitate was allowed to form at room temperature over a 45-min period. Portions representing 0.8 μg of the recombinant DNA were added to cell cultures in 60-mm petri dishes containing 4 ml of fresh medium, and incubation was continued at 37°C for 4 h. After the medium was changed, the cells were treated with 1 ml of 25% dimethylsulfoxide in HBS for 4 min at room temperature. The monolayers were washed again and refed with fresh medium.

Analysis of cellular DNA. Cellular DNA was extracted from confluent monolayers of transformed cells according to a modification of the method of Gross-Bellard et al. (8) as described before (Law et al., in press). Digested DNA was fractionated on 0.6% agarose gel, depurinated and denatured in situ (34), and transferred onto nitrocellulose filters (BA85; Schleicher & Schuell Co.) according to a modification of the procedure of Southern (15, 31).

Radiolabeled DNAs were prepared according to Rigby et al. (29). Hybridization was performed as described previously (Law et al., in press).

Restriction endonucleases were obtained from New England Biolab and used in the recommended buffer system. Analytical and preparative reactions contained 1 U of enzyme per μg of DNA. Incubation was for 60 min at 37°C, after which the enzyme was inactivated (15 min at 68°C).

Preparative and analytical electrophoreses were in Tris-acetate buffer (5 mM sodium acetate, 1 mM EDTA, 40 mM Tris, pH 7.8) at either 2.5 V/cm for 16 h or 100 mA for 4 h. After electrophoresis, the gels were stained with ethidium bromide (0.5 μg/ml), and the DNA was visualized with short-wave ultraviolet light. DNA was recovered from the gel by electroelution into a dialysis bag, extracted with phenol, and precipitated with ethanol in the presence of 200 mM NaCl.

In situ hybridization. Cells were seeded in 60-mm plates. Twenty-four hours later the monolayers were transferred onto nitrocellulose filters, denatured, and hybridized with ^{32}P-labeled DNA probes as described previously (33).

Construction of recombinant DNAs. The steps involved in the construction of the recombinant molecules are diagrammed in Fig. 1. The rat preproinsulin gene I DNA was the generous gift of A. Efstratiadis. The gene was originally isolated as a λ clone (19) from a rat chromosome DNA library (30). A 5.3-kilobase (kb) fragment was purified and cloned in pBR322 at the *Bam*HI site.

Construction of pBR322-insulin recombinant. A 1.62-kb segment containing the coding sequences of the gene, its intervening sequence, and the regulatory signals at the 5' and 3' termini was generated from the cloned 5.3-kb DNA by a *Bam*HI+*Hin*cII digest (Fig. 1). After two purification steps through agarose gels the DNA was electroeluted from the gel, extracted with phenol, and precipitated with ethanol.

^{32}P-labeled synthetic *Hin*dIII linkers (Collaborative Research) were joined to the *Hin*cII site (22), and the products were digested with *Hin*dIII to generate tails with monomeric linkers. Modified 1.62-kb fragments were then ligated to the 4.0-kb fragment of *Bam*HI+*Hin*dIII-cleaved pBR322, and the ligation mixture was used to transform *Escherichia coli* K-12 strain HB101 (13).

Plasmid DNA from ampicillin-resistant, tetracycline-sensitive colonies was isolated (35) after an amplification step with chloramphenicol (4) and analyzed with restriction enzymes for the presence of the 1.62-kb fragment. One such plasmid, prI_1 (1.62 kb), was selected for further study.

Construction of pBR322-bovine papilloma virus insulin recombinant DNA. A recombinant plasmid, $pBPV_{69T}$, containing the 69% transforming region of bovine papilloma virus 1 DNA, has been described (12, 20). Viral DNA was excised from this recombinant by *Bam*HI+*Hin*dIII digestion (Fig. 1), purified on an agarose gel, and ligated to the gel-purified, 1.62-kb fragment of prI_1. After digestion with *Hin*dIII, the products were ligated to *Hin*dIII-cleaved pBR322, and the resulting DNA was used to transform *E. coli* strain HB101. DNA from ampicillin-resistant, tetracycline-sensitive colonies was isolated and analyzed with restriction endonucleases. One of the colonies containing the recombinant DNA ($pBPV_{69T}$-rI_1) was isolated, amplified, and used for subsequent studies. A physical map of the recombinant thus constructed is presented in Fig. 2.

Nuclease S1 mapping. Cytoplasmic ribonucleic acid (RNA) was prepared from approximately 10^8 cells as described previously (16), and the polyadenylic acid-containing fraction was selected by chromatog-

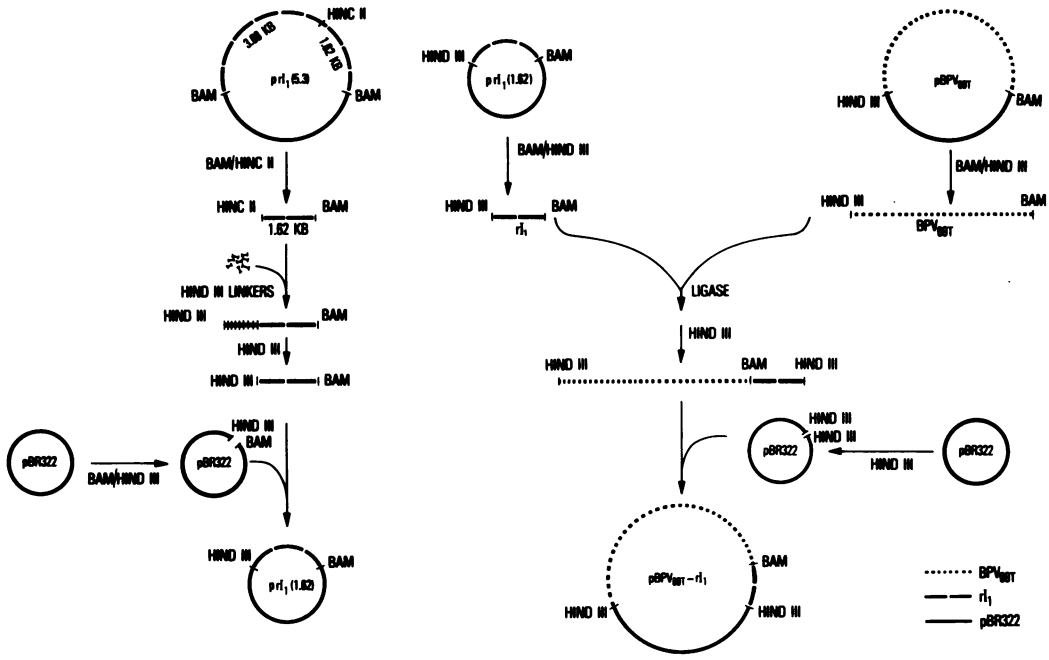

FIG. 1. *Construction of bovine papilloma virus recombinant containing the rat preproinsulin gene I. Symbols: dotted lines, BPV_{69T} sequences; broken lines, rI_1 sequences; solid lines, pBR322 sequences. For details, see text.*

raphy on an oligodeoxythymidylic acid-cellulose column (1).

To obtain ^{32}P-labeled DNA probe, African green monkey kidney cells were coinfected with recombinant SV40 (SVL_1-rI_1) containing the preproinsulin gene (9) and *ts*A28 as a helper and labeled 24 h later with 0.1 mCi of [^{32}P]phosphate (Amersham Corp.) per ml in phosphate-free medium. After a 48-h labeling period, viral DNA was prepared (11), and form I DNA was isolated on a cesium chloride-ethidium bromide equilibrium gradient. The DNA was digested with *Hae*II+*Bam*HI, and the 1,560-base pair fragment containing the preproinsulin gene was purified on an agarose gel and used as a probe.

RNA transcripts were analyzed by the S1 nuclease method of Berk and Sharp (2). Briefly, polyadenylic acid-containing RNA was mixed with 10^4 cpm of [^{32}P]DNA probe (specific activity, 10^6 cpm/μg), the mixture was precipitated with ethanol, and the precipitate was resuspended in 30 μl of formamide buffer [80% formamide, 50 mM NaCl, 50 mM PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)], pH 6.4]. DNA duplexes were dissociated at 68°C for 15 min, the mixture was transferred to a 50°C water bath, and hybridization was continued for 3 h at 50°C. The products were then treated with S1 nuclease (Miles Laboratories) under the conditions described by Berk and Sharp (2). Resulting DNA segments were precipitated with ethanol, resuspended in electrophoresis buffer (30 mM NaOH, 2 mM EDTA), and analyzed on a 1.4% alkaline agarose gel as described previously (24), except that the gel was cast in 30 mM NaCl–0.2 mM EDTA and made alkaline by prerunning it in electrophoresis buffer for 60 min. Electrophoresis was at 40 V for 12 h.

Protein analysis. Cells in 100-mm plates were washed 3 h before labeling with Earle balanced salts containing 5% normal medium and 2% dialyzed fetal bovine serum (GIBCO) and labeled in the same medium with 200 μCi of L-[^{35}S]cysteine per ml (855.6 Ci/mmol; New England Nuclear Corp.) for 4 h at 37°C.

Lysis of cells was performed in 1 ml of Tris-buffered saline (pH 7.6) containing 1% Nonidet P-40, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, and 2 mM *N*-tosylphenylalanine chloromethyl ketone. The proteins were then immunoprecipitated (14) with anti-bovine insulin serum (Miles Yeda, Israel). For competitive binding studies the antiserum was first neutralized with bovine insulin (2 μg of bovine insulin per 6 μl of antiserum, 30 min at 4°C), after which the mixture was added to the samples.

Immunoprecipitated proteins were analyzed on sodium dodecyl sulfate-polyacrylamide gels (17). After electrophoresis, gels were fixed for 60 min in a solution containing 10% trichloroacetic acid–10% glacial acetic acid–30% methanol, impregnated with En^{3}Hance (New England Nuclear Corp.) for 60 min, and then treated with water for another 60 min. The gels were dried and exposed to XR-5 film (Kodak) at −70°C.

RESULTS

Transformation of cells and screening of transformants for bovine papilloma virus and rI_1 sequences. BPV_{69T}-rI_1 DNA was separated from plasmid DNA by *Hin*dIII digestion,

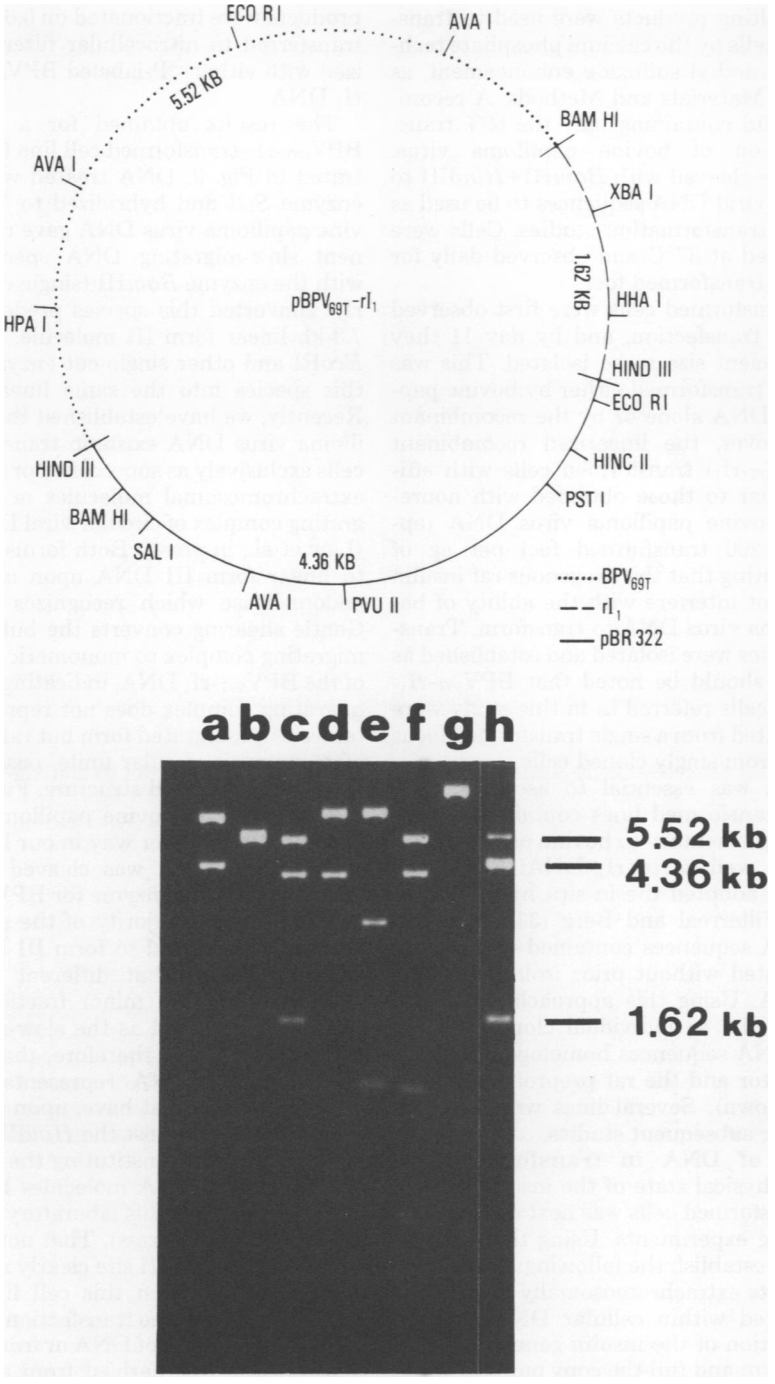

FIG. 2. *Restriction map of pBPV$_{69T}$-rI$_1$ recombinant. The orientation of BPV$_{69T}$ DNA relative to rI$_1$ DNA as well as restriction sites in the molecule are indicated. Below the map is an ethidium bromide-stained 1.2% agarose gel containing restriction fragments of BPV$_{69T}$-rI$_1$ DNA. (a) HindIII; (b) BamHI; (c) BamHI+HindIII; (d) EcoRI; (e) EcoRI+XbaI; (f) HindIII+XbaI+BamHI; (g) XbaI; (h) purified 69% transforming fragment of bovine papilloma virus (5.52 kb) and pBR322 (4.36 kb) and rI$_1$ DNAs (1.62 kb).*

and the resulting products were used to transform mouse cells by the calcium phosphate technique and dimethyl sulfoxide enhancement, as described in Materials and Methods. A recombinant plasmid containing only the 69% transforming region of bovine papilloma virus, $pBPV_{69T}$, was cleaved with *Bam*HI+*Hin*dIII to generate the viral DNA sequences to be used as a control in transformation studies. Cells were then incubated at 37°C and observed daily for formation of transformed foci.

Foci of transformed cells were first observed 7 days after transfection, and by day 11 they were of sufficient size to be isolated. This was true for cells transformed either by bovine papilloma virus DNA alone or by the recombinant DNA. Moreover, the linearized recombinant DNA (BPV_{69T}-rI_1) transformed cells with efficiencies similar to those obtained with nonrecombinant bovine papilloma virus DNA (approximately 200 transformed foci per μg of DNA), indicating that the exogenous rat insulin DNA does not interfere with the ability of bovine papilloma virus DNA to transform. Transformed colonies were isolated and established as cell lines. It should be noted that BPV_{69T}-rI_1-transformed cells referred to in this study were each propagated from a single transformed focus rather than from singly cloned cells.

At first it was essential to ascertain that BPV_{69T}-rI_1-transformed lines contain DNA sequences complementary to bovine papilloma virus DNA as well as to rI_1 DNA. For initial screening we adopted the in situ hybridization method of Villarreal and Berg (33), in which specific DNA sequences contained within cells can be detected without prior isolation of the cellular DNA. Using this approach, we found that each of the 48 individual clones isolated contained DNA sequences homologous to both the viral vector and the rat preproinsulin gene (data not shown). Several lines were selected arbitrarily for subsequent studies.

Analysis of DNA in transformed cell lines. The physical state of the insulin gene in selected transformed cells was next analyzed by DNA blotting experiments. Using this analysis we wished to establish the following: (i) whether the gene exists extrachromosomally or whether it is integrated within cellular DNA; (ii) the physical location of the insulin gene relative to the viral vector; and (iii) the copy number of the recombinant DNA molecule in transformed cells.

Total DNA isolated from transformed cells was either gently sheared or treated with a restriction endonuclease that recognizes no site (*Sst*I) or a single site (*Bam*HI, *Eco*RI, and *Hin*dIII) within BPV_{69T}-rI_1 DNA. Resulting products were fractionated on 0.6% agarose gels, transferred to nitrocellular filters, and hybridized with either ^{32}P-labeled BPV_{69T}-rI_1 DNA or rI_1 DNA.

The results obtained for a representative BPV_{69T}-rI_1-transformed cell line (NS8) are illustrated in Fig. 3. DNA treated with the no-cut enzyme *Sst*I and hybridized to ^{32}P-labeled bovine papilloma virus DNA gave rise to a prominent slow-migrating DNA species. Digestion with the enzyme *Bam*HI (single cut for BPV_{69T}-rI_1) converted this species predominantly to a 7.3-kb linear form III molecule. Cleavage with *Eco*RI and other single-cut enzymes converted this species into the same linear component. Recently, we have established that bovine papilloma virus DNA exists in transformed mouse cells exclusively as supercoiled or nicked circular extrachromosomal molecules or in a slow-migrating complex of circular viral DNA molecules (Law et al., in press). Both forms are converted to linear form III DNA upon cleavage by an endonuclease which recognizes a single site. Gentle shearing converts the bulk of the slow-migrating complex to monomeric forms I and II of the BPV_{69T}-rI_1 DNA, indicating that the slow-migrating complex does not represent a concatameric or integrated form but rather a complex of monomeric circular units, possibly in an intertwined catenated structure. Further study of this structure in bovine papilloma virus-transformed cells is under way in our laboratory.

When the DNA was cleaved with *Hin*dIII (also a single-site enzyme for BPV_{69T}-rI_1 DNA), again the great majority of the slow-migrating band was converted to form III DNA. The results were somewhat different after *Hin*dIII cleavage in that a minor fraction of residual DNA still migrated as the slow-migrating species. We conclude, therefore, that the residual slow-migrating DNA represents recombinant DNA molecules that have, upon circularization after transfection, lost the *Hin*dIII site. Loss of a restriction site constituting the cohesive ends of transfecting DNA molecules has previously been described by this laboratory and by others (18; Law et al., in press). That not all molecules have lost the *Hin*dIII site clearly indicates DNA heterogeneity within this cell line. This may result either from the transfection of a single cell by multiple species of DNA or from the fact that this cell line was derived from a single transformed focus containing perhaps several independently transformed cells rather than from a single transformed cell.

When ^{32}P-labeled rI_1 DNA was used as a probe on duplicate blots (obtained from the same preparation of restricted DNA [Fig. 3]), it annealed to all fragments observed with the ^{32}P-labeled

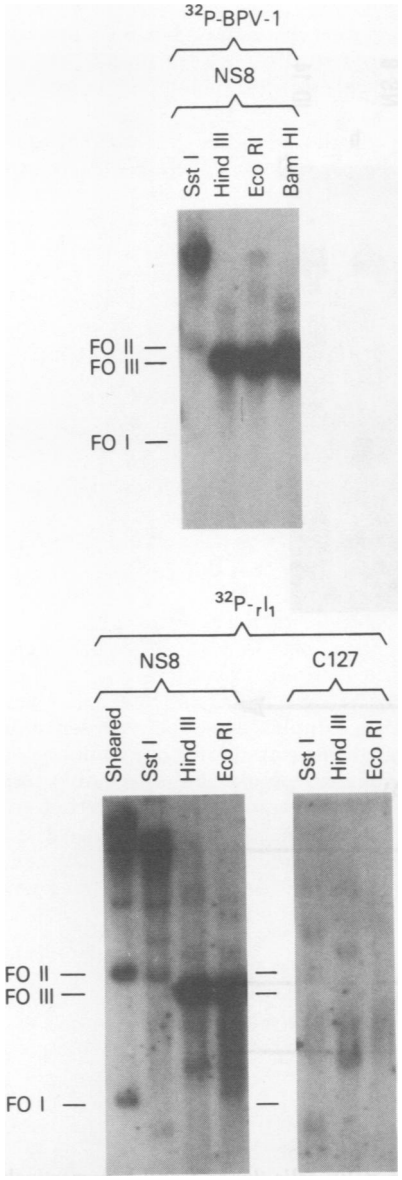

FIG. 3. *Blot hybridization of ^{32}P-labeled bovine papilloma virus 1 DNA and ^{32}P-labeled rI$_1$ DNA to cellular DNAs purified from untransformed C127 cells and from BPV$_{69T}$-rI$_1$-transformed cells (NS8). Cellular DNAs (10 μg) cleaved to completion with the indicated restriction endonucleases were fractionated on 0.6% agarose gels. Gentle shearing was accomplished by passing the DNA through a 23-gauge needle 10 times. The DNAs were depurinated, denatured in situ, and tranferred onto nitrocellulose filters as described in the text. These cellular blots were hybridized to 5 × 10^6 cpm of nick-translated, denatured, ^{32}P-labeled bovine papilloma virus 1 DNA (1.5 × 10^8 cpm/μg) or ^{32}P-labeled rI$_1$ DNA (10^8 cpm/μg) at 60°C for 20 h. Filters were washed extensively, air dried, and exposed to X-ray film for 24 h at −70°C.*

bovine papilloma virus probe. In addition, several DNA species were exposed which did not hybridize with the ^{32}P-labeled bovine papilloma virus probe. Since these same bands were also demonstrated in DNA from untransformed C127 cells with the ^{32}P-labeled rI$_1$ DNA probe (Fig. 3), we conclude that they represent endogenous preproinsulin-related DNA sequences. DNA isolated from untransformed cells did not hybridize with any bovine papilloma virus 1 DNA sequences (data not shown).

In addition to the predominant high-molecular-weight forms described above, several minor DNA species were also observed. Although it is possible that these bands represent integrated DNA forms, we think it unlikely. First, previous studies from this laboratory have established that bovine papilloma virus DNA sequences persist in transformed mouse cells exclusively in a free, nonintegrated episomal form (Law et al., in press). Second, if these minor bands represented integrated sequences, one would expect some variation between the hybridization pattern obtained with the ^{32}P-labeled bovine papilloma virus probe and that using the ^{32}P-labeled rI$_1$ probe (due to size heterogeneity in the flanking host sequences). This is especially true for analyses of *Bam*HI fragments, which separate the recombinant into its bovine papilloma virus and rI$_1$ components (Fig. 1). As we have shown, the pattern obtained with three different endonucleases were identical except for the additional endogenous insulin-related sequences seen with the ^{32}P-labeled rI$_1$ probe.

RNA analysis. To ascertain whether insulin-specific RNA transcripts are produced in BPV$_{69T}$-rI$_1$-transformed cells, we have used the S1 nuclease mapping method of Berk and Sharp (2). In this analysis polyadenylic acid-containing RNA is hybridized to a ^{32}P-labeled DNA probe under conditions which favor the formation of RNA-DNA duplexes. Hybrids thus formed are treated with S1 nuclease, which hydrolyzes the single-stranded DNA tails at the 3′ and 5′ termini of the duplex molecule as well as the unhybridized intervening sequences within the gene. The resulting products analyzed on alkaline agarose gels represent the exons present in specific messenger RNAs (mRNA's).

Figure 4 illustrates the results obtained in such an analysis. When authentic rat preproinsulin mRNA, derived from rat insulinoma cells (3), was hybridized with the ^{32}P-labeled SVL$_1$-rI$_1$ DNA probe, a single band of 402 nucleotides was detected. This band represents the coding sequences at the 3′ end of the insulin gene. The 5′ end of this fragment delineates the 3′ terminus of the excised intervening sequences. The expected 42-nucleotide leader sequence is not re-

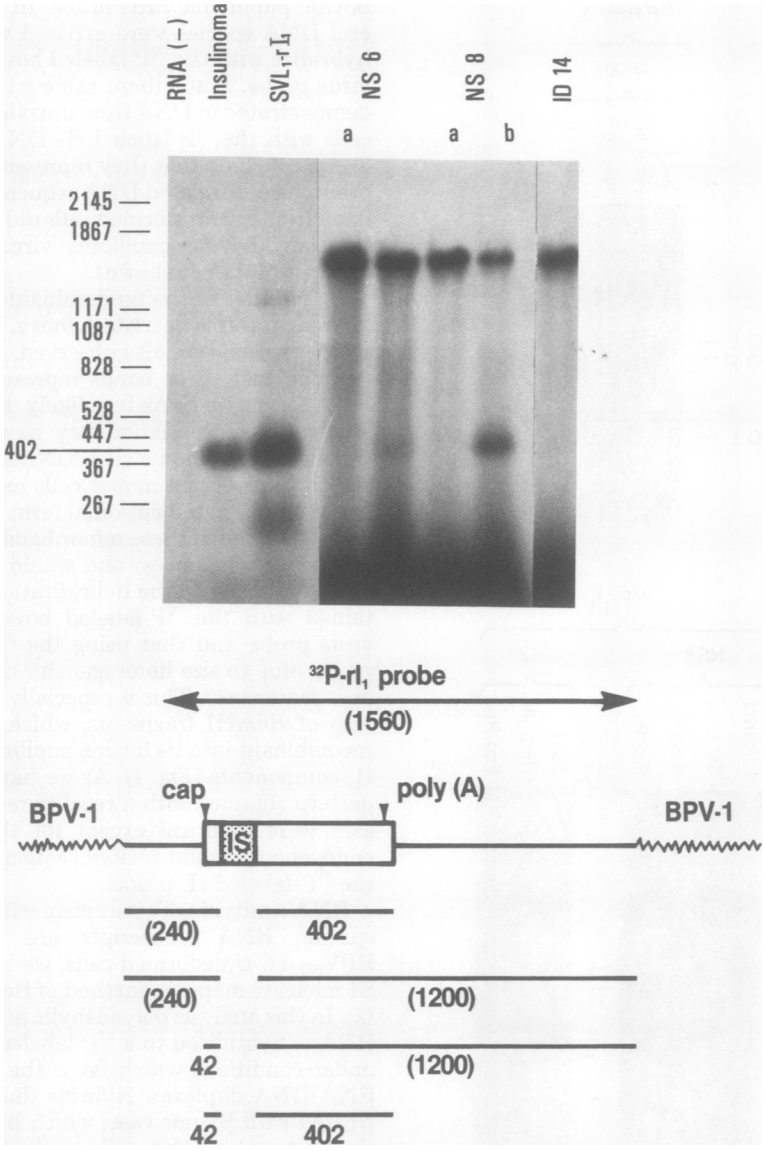

FIG. 4. *Polyadenylic acid-selected RNA from 2 × 10⁶ and 5 × 10⁶ cells (lanes a and b, respectively) were mixed with 10,000 cpm of ³²P-labeled rat preproinsulin DNA (2 × 10⁶ cpm/μg) purified from recombinant SVL1-rI1 virus by HaeII+BamHI digestion. The mixture was precipitated with ethanol, resuspended in 30 μl of formamide buffer, and hybridized for 3 h at 50°C. RNA-DNA duplexes were treated with S1 nuclease, and the digests were analyzed by electrophoresis through a 1.4% alkaline agarose gel. The gel was exposed to XR5 film (Kodak) for 72 h at −70°C. Numbers to the left of the gel indicate the size, in base pairs, of SV40 DNA segments. The 1,560-base species represent the self-annealed ³²P-labeled DNA probe. DNA protected by authentic preproinsulin mRNA is 402 base pairs long. RNA(−), Probe contained no added RNA; insulinoma, RNA from rat insulinoma cells; SVL1-rI1, RNA from African green monkey kidney cells infected with SV40-insulin recombinant DNA; NS8, RNA from BPV69T-rI1-transformed cells; ID14, RNA from virally transformed cells. Diagram depicts all the classes of DNA fragments expected if both viral and insulin promoters are involved in transcription.*

solved in the gel system used. RNA from BPV_{69T}-rI_1-transformed cell lines (NS6 and NS8) similarly analyzed also protected a single 402-base DNA fragment (Fig. 4). This band was seen in every BPV_{69T}-rI_1-transformed cell line tested. In contrast, control RNA from bovine papilloma virus-transformed cells (ID14) did not protect this DNA fragment (Fig. 4). Since the

size of the DNA fragments obtained in these two cases is identical and since the 5′ end is fixed by the splice junction, we conclude that the entire coding region of the preproinsulin gene is represented in mRNA produced in BPV_{69T}-rI_1-transformed cells. We also infer from these observations that the polyadenylation signal at the 3′ end of the gene is faithfully recognized.

For comparison, the pattern obtained with RNA from African green monkey kidney cells infected with SV40-insulin recombinant virus (9) is included. In this case three predominant DNA fragments, consisting of 402, 240, and 1,420 nucleotides, are detected (9). The 402-nucleotide fragment corresponds to the 3′ exon of the authentic rat preproinsulin mRNA; the 240-nucleotide fragment is derived from a transcript that uses an SV40 promoter and extends to the 5′ end of the preproinsulin gene splice site; and the 1,420-nucleotide fragment is derived from a transcript which utilizes a polyadenylation site located within SV40 sequences (9).

The presence of only one insulin-specific transcript, corresponding to the coding sequences of the gene, in BPV_{69T}-rI_1-transformed cells suggests that viral termination signals are not involved in the transcription of rat preproinsulin gene. Preliminary data from S1 nuclease and exonuclease VII analyses indicate that the detectable transcripts in BPV_{69T}-rI_1-transformed cells initiate at a promoter within rat insulin sequences. A detailed analysis of the 5′ end is in progress.

Protein analysis. After the insulin-specific RNA in BPV_{69T}-rI_1-transformed cells was identified, the efficacy of the transcripts in directing translation and processing of polypeptides was investigated. The production of insulin from its primary translational products involves several discrete steps. First, preproinsulin, a 110-amino acid polypeptide consisting of a hydrophobic preregion plus the "B," "C," and "A" chains, is produced. The prehormone is then transferred through the microsomal membrane and the preregion peptide is cleaved off, thus generating the proinsulin peptide. Subsequent folding of the proinsulin molecule brings the A and B chains into close proximity to allow the formation of disulfide bridges between the two chains. Once the disulfide bonds have been formed, the internal C chain is removed, thereby converting proinsulin to insulin.

We used a quantitative radioimmunoassay to screen tissue culture media from several cell lines for the presence of a protein bearing insulin-specific determinants. Media from BPV_{69T}-rI_1-transformed cells contained from 10 to >400 μU (1 U = 48 ng) of material per ml immunoreactive with anti-insulin serum (Table 1). This represents a 2- to 80-fold increase over the 5 μU/ml present in medium from untransformed C127 cells. When the medium of cells transformed by BPV_{69T} alone was analyzed, only background levels (<6 μU/ml) were detected. This indicates that secretion of insulin or insulin-like material is not a property of bovine papilloma virus-transformed cells in general, but rather a function of the exogenous DNA used in transformation.

The identity of the protein was established as rat proinsulin by competitive immunoprecipitation studies followed by analysis on sodium dodecyl sulfate-polyacrylamide gels. Cells were labeled with [^{35}S]cysteine, and an extract was prepared and immunoprecipitated with hamster anti-bovine insulin serum. As a reference, the pattern obtained with a lysate of African green monkey kidney cells infected with SV40-rat preproinsulin recombinant virus ($SVL1$-rI_1) (9) is presented (Fig. 5A). In this case a proinsulin polypeptide is immunoprecipitated which shows a migration pattern similar to that of authentic bovine proinsulin marker. (Bovine proinsulin migrates slightly ahead of rat proinsulin due to its smaller size.) Analysis of BPV_{69T}-rI_1-transformed cells [NS6(−), NS8(−), and NS24(−)] demonstrated the presence of a prominent band identical in its electrophoretic mobility to proinsulin from SVL_1-rI_1-infected cells (Fig. 5A). This band was absent from the lysates prepared from cells transformed by bovine papilloma virus alone (ID14). In the competitive binding study, samples were incubated with antiserum previously neutralized with 2 μg of bovine insulin. Under these conditions, no proinsulin-like protein was immunoprecipitated [NS6(+), NS8(+), and NS24(+)].

To demonstrate the secretion of proinsulin into the medium, analyses similar to those performed on the cell extracts were made on culture medium from BPV_{69T}-rI_1-transformed cells. A distinct band, the size of rat proinsulin, was

TABLE 1. *Secretion of insulin-like material from transformed and untransformed mouse cell lines*

Mouse cell line	Insulin-like material secreted in 24 h by 10^6 cells (μU/ml)[a]	No. of lines/ total	%
Transformed by BPV_{69T}-rI_1	>400	24/48	50
	200–400	11/48	23
	40–200	10/48	21
	10–40	3/48	6
Transformed by BPV_{69T}	<5	6/6	100
Untransformed C127	<5	6/6	100

[a] Rate of insulin-like material production for a representative BVP_{69T}-rI_1-transformed cell line (NS8) = 400 μU/12 h per 10^6 cells.

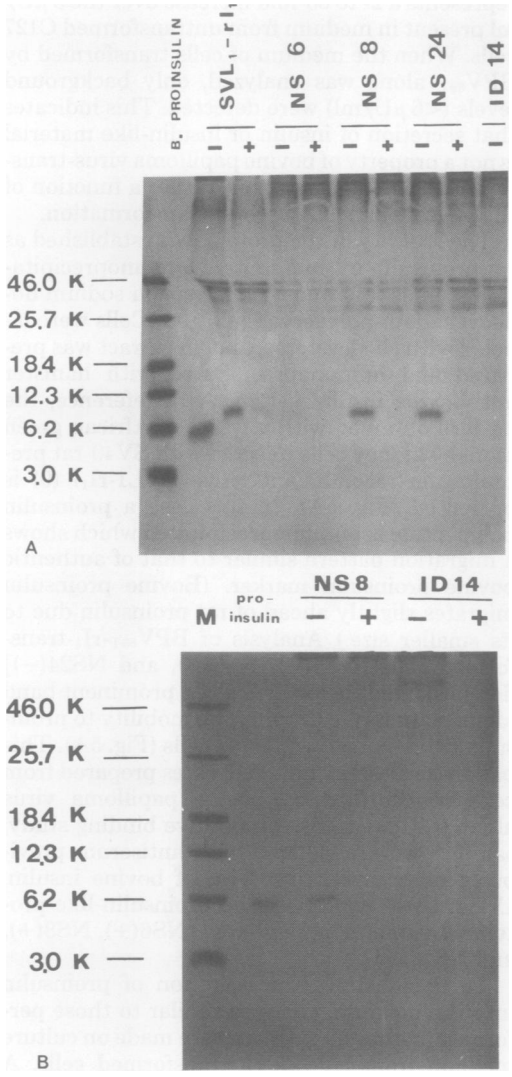

FIG. 5. *Cells in 100-mm plates were labeled with 715 μCi (200 μCi/ml) of [^{35}S]cysteine for 4 h at 37°C, and the proteins were recovered as described in the text. Samples equivalent to medium from 10^6 cells or extracts from 1.5×10^6 cells were immunoprecipitated with hamster anti-bovine serum for 16 h at 4°C. In competitive binding studies the antiserum was first neutralized with 2 μg of bovine insulin (30 min on ice) before it was added to the samples. Immunoprecipitated proteins were analyzed on sodium dodecyl sulfate-polyacrylamide gels. NS6, NS8, and NS24 are cells transformed by BPV$_{69T}$-rI$_1$ DNA. ID14 are cells transformed by bovine papilloma virus. (−) No competition; (+) with competition. (A) Analysis of cellular extracts on a 10 to 17% polyacrylamide linear gradient gel. (B) Analysis of proteins secreted into the medium on a linear 16% polyacrylamide gel. Migration of ^{14}C-labeled markers and ^{14}C-labeled bovine proinsulin is indicated.*

precipitated with anti-bovine insulin serum [NS8(−)] but not with neutralized serum [NS8(+)] (Fig. 5B). Medium from cells transformed by bovine papilloma virus alone (ID14) did not contain this protein.

These results indicate that insulin-specific RNA molecules produced in BPV$_{69T}$-rI$_1$-transformed cells are translated and that processing of these proteins results in the formation of proinsulin, which is secreted into the medium.

DISCUSSION

Previous studies have established that mouse cells transformed by bovine papilloma virus 1 contain viral DNA sequences which exist exclusively in a free extrachromosomal state (Law et al., in press). This is true for cells transformed by the intact virus, by cloned linearized viral DNA, or by a cloned 69% subgenomic DNA fragment. The unique ability of the papilloma viruses to transform cells in the absence of integration prompted us to assess the potential use of bovine papilloma virus 1 DNA as a eucaryotic cloning vector.

Our experimental design consisted of the construction of a recombinant DNA molecule containing the 69% transforming region of bovine papilloma virus 1 DNA and the rat preproinsulin I gene, which contains all of the regulatory signals (putative promoter, polyadenylation site, and intervening sequences) necessary for faithful transcription.

Mouse cells transformed by the recombinant molecules were isolated and tested for the expression of the exogenous gene. Using several criteria, we have demonstrated that (i) preproinsulin DNA sequences exist predominantly if not exclusively as free nonintegrated episomes within transformed cells; (ii) the exogenous gene is transcribed into mRNA similar if not identical to authentic preproinsulin mRNA; (iii) these transcripts direct the synthesis of proinsulin protein; and (iv) the gene product is secreted by the cells into the tissue culture medium in large amounts.

It is noteworthy that 100% of the transformed cell lines tested (48 of 48) contained both bovine papilloma virus and rI$_1$ DNA sequences. Since these cell lines were initially selected solely on the basis of their ability to grow as foci in an untransformed cell monolayer, it seems that transformation per se is a sufficient criterion for the isolation of cells that have stably incorporated the exogenous DNA.

Analysis of the DNA in transformed cells demonstrated the presence of multiple copies of BPV$_{69T}$-rI$_1$ DNA existing predominantly if not exclusively in a nonintegrated episomal state.

Based on reconstruction experiments, we estimated that each cell contains an average of 60 to 80 copies of recombinant DNA molecules. In an earlier communication (Law et al., in press) we have shown that cell lines transformed by bovine papilloma virus DNA alone contain 19 to 118 viral copies per diploid cell genome. Since the value obtained in this study falls within this range, it appears that exogenous DNA sequences do not impede the replication of the viral episome.

S1 nuclease analysis of RNAs produced in BPV_{69T}-rI_1-transformed cells revealed the presence of only one preproinsulin-specific RNA representing the entire coding region of the gene. This finding differs from those obtained in another viral-vector system in which the rat preproinsulin gene was inserted into the late region of SV40 DNA. Cells infected with the SVL_1-rI_1 recombinant contained several preproinsulin RNA species, only one of which corresponded to authentic mRNA. Other transcripts were derived from both initiation and termination at viral regulatory sequences (9). Similarly, the mouse globin gene covalently linked to SV40 DNA is transcribed from either its own promoter or the viral late region promoter (10). Preliminary evidence using exonuclease VII in conjunction with S1 nuclease analysis indicates that all of the detectable transcripts produced in BPV_{69T}-rI_1-transformed cells are initiated from the preproinsulin promoter. A detailed study of the 5′ end is in progress. If indeed the bovine papilloma virus promoter does not initiate transcription of foreign DNAs, then this system would be ideal for localizing regulatory elements of various genes and for assessing the effects of induced and naturally occurring mutation on promoter function(s).

We have also demonstrated that substantial levels of proinsulin protein are synthesized in transformed cells and secreted into the medium. Insulin, normally produced in the β cells of the pancreas, is first synthesized as preproinsulin. Two post-translational processing events, the removal of the leader sequence followed by removal of the internal C peptide, convert the preproinsulin to proinsulin and insulin, respectively. The presence of proinsulin in BPV_{69T}-rI_1-transformed cells thus indicates that at least the first processing event occurs in these cells. The apparent absence of the second processing event is probably due to the absence of the processing enzymes necessary for the conversion of proinsulin to insulin.

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