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Expression from an Internal AUG Codon of Herpes Simplex Thymidine Kinase Gene Inserted in a Retrovirus Vector

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We identified structural features that affect the expression of an exogenous gene inserted into a retrovirus vector constructed by using spleen necrosis virus, an avian retrovirus. The thymidine kinase gene from herpes simplex virus type 1 containing deletions in the promoter and terminal sequences of the mRNA was inserted into spleen necrosis virus. We found that synthesis of thymidine kinase by the recovered virus was apparently initiated from internal AUG residues. At least in some cases, however, the level of expression depended on the number of AUGs and the nucleotide sequence around the AUGs that preceded the initiator codon of the thymidine kinase gene.

Retroviruses are single-stranded RNA viruses that replicate through an obligatory DNA intermediate (3, 25). This DNA intermediate is integrated into the host genome and is propagated as a cellular genetic element. This property makes retroviruses an attractive vector for the introduction and stable maintenance of exogenous DNA in vertebrate cells. Various laboratories have used retroviruses to this end (14, 17, 23, 28). Our laboratory has used spleen necrosis virus (SNV) as a vector. SNV is an avian retrovirus which can infect mammalian cells (rat and dog) at high efficiency.

Retrovirus genes are expressed both from full-length viral RNA and from spliced messages transcribed from a promoter in the viral long terminal repeat (LTR). The AUG used for the initiation of viral protein synthesis can vary. In SNV, the first AUGs from the 5′ end of the mRNA are used for *gag* and for *env* (13; unpublished data), whereas the Prague C strain of Rous sarcoma virus uses the fourth AUG as an initiator codon (15).

We wanted to study some of the parameters that control expression of exogenous genes inserted in an SNV vector. We chose to study the herpes simplex virus (HSV) thymidine kinase (*tk*) gene. The HSV *tk* gene has been sequenced and is well characterized. We removed the HSV *tk* promoter and sequences for the 3′ end of the normal *tk* mRNA from our constructs (17). Since we know both the HSV *tk* gene and the 5′ SNV sequences, we were able to study the effect of additional translational start codons preceding the normal start codon of the *tk* gene on the expression of the inserted HSV *tk* gene.

(Marsden et al. [10] have recently presented evidence that translation of *tk* mRNA is initiated at three separate AUG codons. However, the biological activity of two of these proteins which are initiated from internal AUG residues has not been determined.)

In all cases we looked at the activity of proviral DNA, that is, DNA integrated in chromosomes at a low copy number. We also normalized our results to the physical numbers of vector viruses.

It has been proposed that the first AUG in a message is translated if it is in the appropriate consensus sequence ANNAUGN or GNNAUGPu (8). This principle, though of wide applicability, has several exceptions. Two initiation codons separated by four nucleotides giving rise to separate polypeptides in influenza B virus have been found by Shaw et al. (16). An mRNA from the E1b region of adenovirus contains two initiator AUG codons from which two tumor antigens are translated from different reading frames (2). Some simian virus 40 recombinant DNA vectors containing both the agnoprotein AUG and a foreign gene AUG initiate protein synthesis at either AUG residue (5, 6, 9).

We found that the synthesis of thymidine kinase (TK) could also be initiated at internal AUG residues in SNV vectors.

MATERIALS AND METHODS

Cells. Chicken cells were from SPAFAS embryos (Norwich, Conn.). Chicken TK$^-$ cells were from M. Wigler; buffalo rat liver (BRL) TK$^-$ cells were from R. Axel; and mouse LMTK$^-$ cells were from R. Kucherlapati. Chicken fibroblasts were grown in Eagle medium containing 20% tryptose phosphate broth, 2% calf serum, and 2% fetal bovine serum; chicken TK$^-$ cells were grown in Temin-modified Eagle medium containing 10% calf serum and 1% chicken serum; BRL TK$^-$ cells were grown in Eagle medium with 7% calf serum; and mouse LMTK$^-$ cells were grown in Eagle medium with 5% fetal bovine serum.

Transfection. Chicken, BRL TK$^-$, and mouse LMTK$^-$ cells were transfected by the calcium phosphate coprecipitation technique described by Graham and van der Eb (4). Salmon sperm DNA (10 μg/ml) was used as a carrier for chicken cells, and calf thymus DNA (20 μg/ml) was used for rat and mouse cells. Rat and mouse cells were treated for 45 s with 15% glycerol in 1× HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer 5 h after transfection (22). For isolation of virus, chicken cells were cotransfected with pSW253 (27), a plasmid that contains infectious reticuloendotheliosis virus strain A as helper. Supernatant medium containing virus was harvested 3 to 5 days after transfection.

Virus harvested after transfection of chicken embryo fibroblasts with a plasmid pPBx is designated PBx in the paper.

Infection. Chicken, chicken TK$^-$, and BRL TK$^-$ cells were infected as described previously (24). For chicken cells, infection was carried out in the presence of 10 μg of Polybrene per ml; for rat cells, 100 μg/ml was used.

Selection of transformants. One day after infection or

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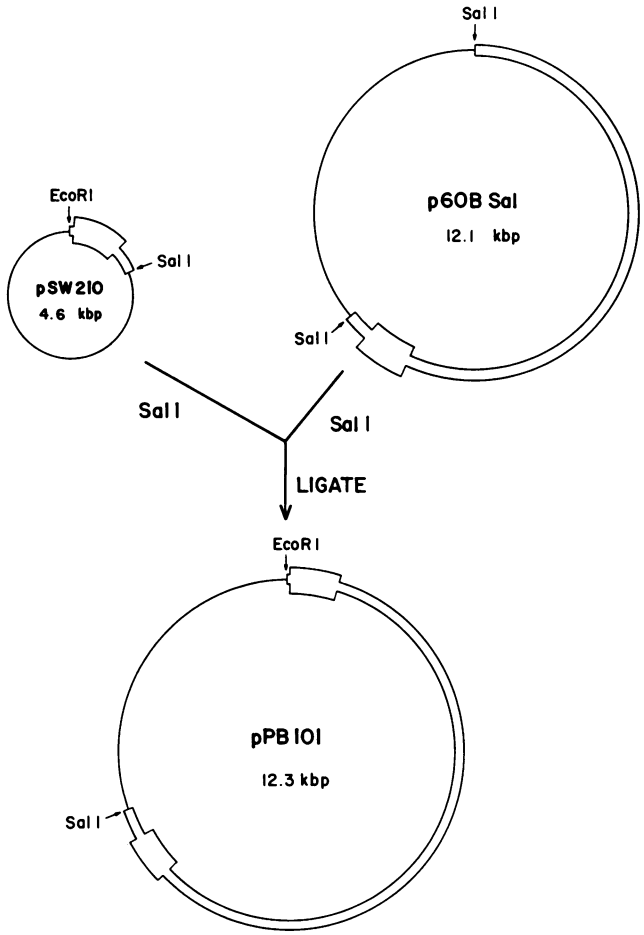

FIG. 1. Construction of pBP101. Constructions were performed as described in the text. Open bars are SNV sequences; the large open boxes are LTRs; and the single lines are pBR322.

transfection, the medium was changed to medium containing 5×10^{-7} M methotrexate, 10^{-4} M hypoxanthine, and 3×10^{-5} M thymidine. The number of drug-resistant colonies was determined after 5 days. This number is denoted as TK-transforming units (TKTU) in this paper. Experiments were done in duplicate. The variation in the number of colonies was within 10% of the number of colonies on the plate.

Construction of SNV-HSV *tk* plasmids. Plasmids pGT6 and pGT2 were obtained from W. G. Tarpley. They are SNV-HSV *tk* recombinant plasmids in which the HSV *tk* gene lacking the promoter and terminal sequences of the mRNA is inserted either at the *Bgl*II or *Bam*HI sites located at 2.0 kilobase pairs (kbp) and 3.6 kbp, respectively, from the 5′ terminus of the viral DNA. The initiator codon, ATG, for the *gag* protein is substituted by ATA.

Plasmids pSW210 (27) and p60B Sal (12) were digested with *Sal*I (Fig. 1). A mixture of the digests was ligated and used to transform *Escherichia coli* HB101 cells. Plasmids from ampicillin-resistant colonies were isolated by the sodium hydroxide-sodium dodecyl sulfate rapid lysis procedure of Birnboim and Doly (1) and were screened for pPB101 by appropriate restriction enzyme digestions.

Plasmid pPB101 was digested with a combination of *Bgl*II and *Eco*RI to obtain the *Eco*RI to *Bgl*II fragment of SNV DNA (0 to 2.0 kbp) containing the wild-type initiator ATG for the *gag* protein (see Fig. 2). A fivefold molar excess of this DNA was mixed with a *Bgl*II and *Eco*RI digest of pGT6 and ligated. This DNA was used to transform *E. coli* HB101 cells. Plasmids were isolated from the ampicillin-resistant colonies by the sodium hydroxide-sodium dodecyl sulfate rapid lysis procedure. Colonies containing pPB160 (Fig. 2) were identified by analysis of the plasmids after digestion with *Bgl*II and *Eco*RI.

Plasmid pPB160 was digested with *Xba*I to delete from 1.02 to 1.95 kbp of SNV to obtain pPB158. Assuming that transcription initiates within the LTR, this deletion moves the *tk* coding sequences closer to the 5′ end of the mRNA.

Plasmid pPB158 was digested with *Bgl*II, and the single-stranded ends were repaired with the Klenow fragment of *E. coli* DNA polymerase I and ligated. pPB166 was obtained after transformation and characterization. In pPB166, *gag* and *tk* are in a continuous reading frame, resulting in the possibility of formation of a *gag-tk* fusion protein.

Plasmid pPB158 was digested with either *Bgl*II or *Xba*I. The single-stranded ends were repaired with the Klenow fragment of *E. coli* DNA polymerase I, and *Cla*I linkers were ligated to the blunt ends. The *Cla*I linker includes an ATG triplet which is thus inserted upstream from the initiator codon of the *tk* gene. The DNA was then religated to give closed circles. Plasmids isolated from ampicillin-resistant colonies obtained after transformation of *E. coli* HB101 cells were characterized. The number of *Cla*I linkers was determined by successively digesting pPB165 with *Sst*I and *Xba*I and pPB168 with *Bgl*II and *Bam*HI and then analyzing by electrophoresis in a 10% polyacrylamide gel with pBR322 digested with *Hpa*II and *Hae*III as molecular weight markers. The structures of pPB165 and pPB166 were also confirmed by nucleic acid sequencing (11). pPB165 has four *Cla*I linkers at the *Bgl*II site, and pPB168 has eight *Cla*I linkers at the *Xba*I site.

Isolation and analysis of unintegrated viral DNA. Unintegrated viral DNA was isolated according to the procedure of Hirt (7). The DNA, either undigested or digested with restriction endonucleases, was electrophoresed in a 1% agarose gel, transferred to nitrocellulose paper, and hybridized to appropriate probes according to methods described by Southern (20). The intensities of the various bands in an autoradiogram were quantified by densitometry with a Beckman DU-8 scanning spectrophotometer.

Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, and the enzyme digestions were carried out according to conditions described by the manufacturer.

*Cla*I linkers, CATCGATG, were from P-L Biochemicals.

RESULTS

We studied expression of the HSV *tk* gene inserted in SNV-based retrovirus vectors. Since the promoter for the *tk* gene was deleted, transcription of the gene had to initiate in the 5′ LTR which contains the promoter sequences for the transcription of viral genes. The aim of the study was to determine the effect on the expression of the *tk* gene of additional ATGs 5′ to the ATG corresponding to the initiator codon of the gene.

Insertion of *tk* genes at different positions. We constructed several SNV-HSV *tk* recombinants differing in the position of the *tk* coding sequences with respect to the 5′ LTR. In all cases, the viral sequences 3′ to the *tk* gene were the same. Figure 3 presents maps of these recombinant viruses. Table 1 shows the number of ATGs preceding the initiator ATG of the *tk* coding sequence. In GT6, the initiator codon for the

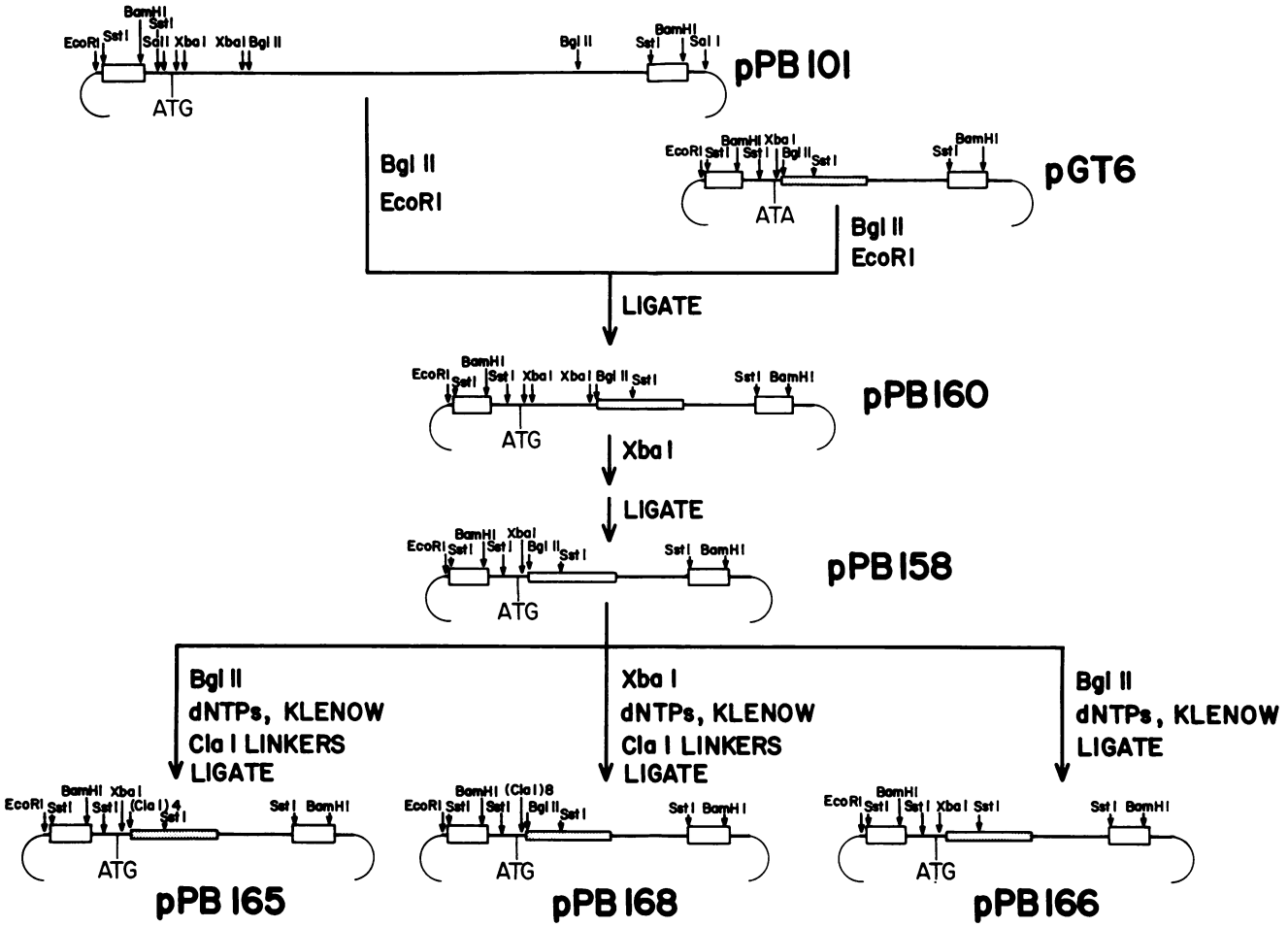

FIG. 2. Construction of pPB158, pPB160, pPB165, pPB166, and pPB168. The dotted boxes represent the sequences for the *tk* gene from HSV. The promoter and terminator regions in the HSV *tk* gene have been deleted (*tk*ΔproΔterR). The open boxes represent the LTRs, and the heavy lines represent other SNV sequences. The pBR322 sequences (thin lines) are only partially shown.

gag protein was replaced by ATA derived from a mutant of SNV (13). Therefore, the ATG of the *tk* gene is the first AUG in the transcript. PB158 retains the initiator codon of the *gag* gene, resulting in one ATG preceding the initiator ATG of the *tk* gene. Although the synthesis of TK from GT6 has to start at the initiator codon for *tk* mRNA because it is the first AUG of the transcript, TK synthesis from PB158, PB160, and GT2, which have additional ATGs 5′ to the first ATG of the *tk* gene, may or may not initiate at this site.

Plasmids containing these constructs were used for transfection of chicken embryo fibroblasts in the presence of helper virus DNA. Virus produced 5 days after transfection was harvested and assayed for TK-transforming activity and for preparation of unintegrated viral DNA. Results of the assay of TKTU are shown in Table 1. Virus recovered from all the plasmids except pGT2 was capable of transforming BRL TK^- cells to the TK^+ phenotype. However, the TKTU titers of PB158, which has one ATG, and PB160, which has thirteen ATGs before the HSV *tk* initiator ATG, were one-half and 1/100, respectively, of GT6, in which there was no ATG 5′ to the initiator ATG for TK.

TKTU, however, is not a direct measure of the absolute amount of TK synthesized in the cell. Rather, it is a reflection of the number of infected TK^- cells converted to a TK^+ phenotype. Based on the results of Spandidos and Wilkie (21), we assume that TKTU is an accurate reflection of the level of *tk* expression.

The amount of unintegrated viral DNA present in an infected cell is a reflection of the efficiency of virus production. We quantified the amount of unintegrated viral DNA synthesized 3 days after infection with each of these recombinant viruses (Fig. 4). The unintegrated viral DNAs ob-

TABLE 1. Specific TKTU of recombinant SNV-HSV *tk* virus

Virus	Relative TKTU	Relative virus production[a]	Specific TKTU[b]	No. of ATGs preceding ATG of *tk* gene
GT6	1	1.0	1.0	0
PB158	0.5	1.25	0.4	1
PB160	0.1	2.33	0.004	13
GT2	<0.0001	0.11	<0.001	>13[c]

[a] Relative amount of recovered viral DNA (recombinant virus/helper virus). Data are from Fig. 4.

[b] TKTU of recombinant virus relative to GT6/relative virus production.

[c] The sequence at the region of *tk* insertion is not known. Therefore, the exact number of ATGs preceding the ATG of the *tk* gene is not known precisely.

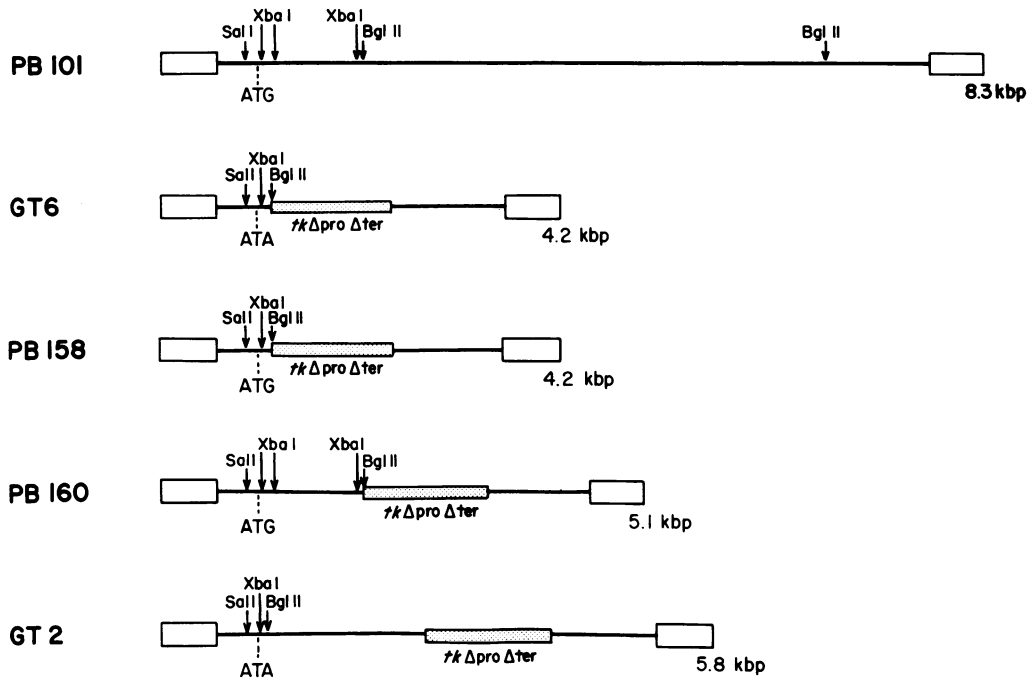

FIG. 3. Structure of SNV-HSV *tk* recombinant viruses. PB101, A schematic of unintegrated viral DNA recovered from infectious plasmid pPB101. The open boxes at the ends represent the LTRs. ATG is the location of the initiator codon for synthesis of the *gag* proteins. The other drawings are of modified viruses containing deletions of viral DNA and insertion of the *tk* gene. The dotted boxes show the location of the *tk* gene (*tk*ΔproΔterR) at different positions in the recombinant viral DNAs. Some of the recognition sites for cleavage by restriction endonucleases have also been indicated.

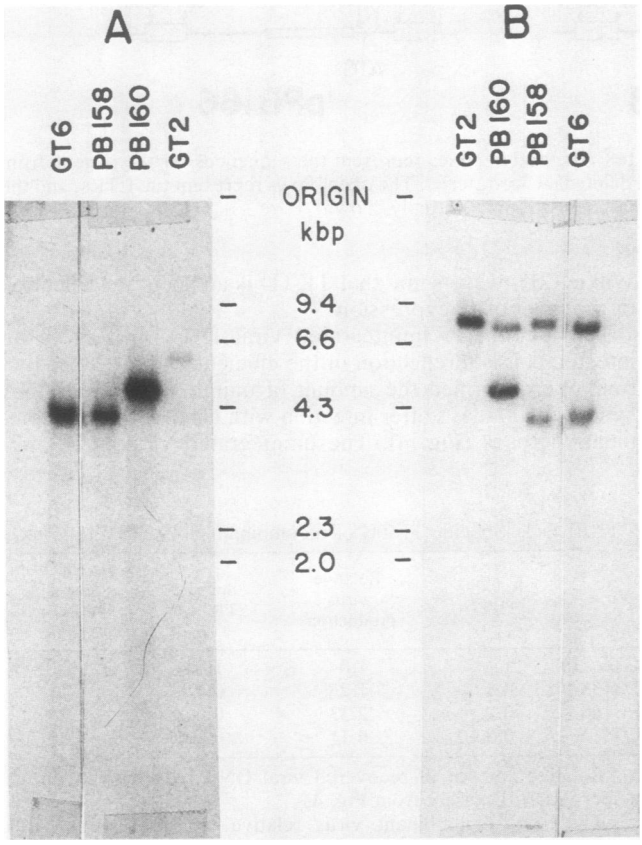

FIG. 4. Unintegrated viral DNA in chicken embryo fibroblasts infected with virus recovered from chicken embryo fibroblasts transfected with SNV-HSV *tk* recombinant plasmids in the presence of plasmid containing helper reticuloendotheliosis virus strain A DNA. The structures of the viral DNAs are shown in Fig. 3. (A) Unintegrated viral DNA was hybridized to ^{32}P-HSV-*tk* DNA. (B) The nitrocellulose paper used in (A) was rehybridized to ^{32}P-SNV DNA.

tained from GT6, PB158, PB160, and GT2 were of the sizes predicted from the recombinant plasmids used to produce them. Unintegrated viral DNA was produced in comparable amounts by GT6, PB158, and PB160, whereas GT2 produced only low amounts of viral DNA (Table 1).

We quantified the TK-transforming activity of these viruses relative to the SNV-*tk* DNA present in the infected cells (Table 1). The specific TKTU observed decreased as the number of ATGs before the first ATG of the *tk* gene increased.

We examined the possibility that the TK-transforming ability of PB160 arose in a minor population of the virus after deletions in the SNV region 5′ to the coding sequences. Virus was recovered after transfection of chicken embryo fibroblasts with pPB160 and was used to transform chicken TK^- cells to the TK^+ phenotype. The virus obtained from a clone of TK^+ cells was used to infect chicken embryo fibroblasts. The unintegrated viral DNA from these cells was examined. No change in the size of the virus DNA was detected (data not shown). Thus, it appears that the TK-transforming activity of PB160 is not the result of deletions in the virus.

Insertion of different numbers of ATGs in front of the *tk* gene. In the above experiments, both the number of ATGs and their distances from the initiator ATG of TK varied. To study the effect of the number of ATGs at a relatively fixed distance from the initiator ATG, we constructed recombinant viruses in which we inserted additional ATGs as part of

a synthetic oligonucleotide. This insertion was accomplished by ligation of commercially available *Cla*I linkers (CATCGATG) at the *Xba*I or *Bgl*II site (located at 1.02 or 1.1 kpb, respectively) in pPB158 (Fig. 2). Four *Cla*I linkers were inserted at the *Bgl*II site in pPB165, and eight *Cla*I linkers were inserted at the *Xba*I site in pPB168.

The TK-transforming activities of the viruses obtained from these constructs were determined. Table 2 summarizes the relative TKTU of the various *tk* recombinant viruses and the number of ATGs preceding the *tk* initiation codon. PB168 had the same relative TKTU as PB158, whereas PB166 and PB165 had only 1/10 as much.

We also determined the amounts of unintegrated SNV-*tk* DNA present in cells infected with virus derived from pPB158, pPB165, pPB166, and pPB168. Equivalent amounts of unintegrated SNV-*tk* DNA were observed in these cells (data not shown). Thus, the differences in biological activity of these recombinant viruses cannot be ascribed to differences in the amount of virus.

TABLE 2. Relative TKTU of virus recovered from different SNV-HSV *tk* plasmids as a function of the number of ATGs preceding the *tk* initiator codon

Clone	No. of ATGs upstream from the *tk* gene			Relative TKTU of recovered virus
	From initiator of the *gag* gene	From *Cla*I linker	Other	
pGT6	0	0	0	1
pPB158	1	0	0	0.5
pPB166	1	0	0	0.05
pPB165	1	4	0	0.05
pPB168	1	8	0	0.5
pPB160[a]	1	0	12	0.01
pGT2[a]	1	0	>12[b]	<0.0001

[a] Data obtained from Table 1.

[b] The sequence at the region of insertion is not known. Therefore, the exact number of ATGs preceding the HSV *tk* initiation codon could not be determined.

DISCUSSION

We studied the effect of insertion of multiple ATG residues 5′ to the initiation codon for protein synthesis of a gene inserted in a retrovirus vector whose own genes use the first AUG in the mRNAs as an initiation codon. Table 2 summarizes the effect of the presence of multiple ATG residues 5′ to the initiator codon for the HSV *tk* gene on the expression of this gene as reflected in the TKTU of virus recovered after transfection.

The number of TKTU has been normalized for the amount of virus recovered as determined by quantifying the amounts of unintegrated viral DNA. The number of TKTU, thus, reflects the activity of *tk* genes inserted in chromosomal DNA by a retrovirus vector. Others have previously shown using transfection assays that the number of stable TK transformants reflects the activity of the *tk* gene in an early expression assay (21).

Expression of TK activity from an integrated retrovirus vector can occur in the following ways: (i) synthesis of a *gag-tk* fusion protein with TK activity, (ii) loss of 5′ AUG codons by mutation, (iii) loss of 5′ AUG codons by splicing at cryptic splicing sites, and (iv) internal initiation at the initiation codon for the *tk* gene. Under hypotheses i, ii, and iii, initiation of TK protein synthesis takes place at the first AUG in the transcript. Under hypothesis iv, initiation of TK protein synthesis takes place at an internal AUG codon.

Examination of the DNA sequence at the SNV-*tk* junction (13, 26) indicates that protein synthesis starting at the initiation codon for *gag* synthesis would be prematurely terminated as a result of termination codons in the *tk* insert in all constructs except PB166. The efficient expression of TK activity by PB158 and PB168 as reflected in their TKTU is inconsistent with hypothesis i. However, the reduced activity of PB166 may be explained by this hypothesis if it is asssumed that the *gag-tk* fusion protein has reduced TK activity.

Under hypothesis ii, a mutation would remove upstream ATG codons. Both PB158 and PB168 have high TK-transforming activity. The removal of the ATG codons would have to occur at the improbable rate of 50% to account for the TKTU which are one-half the value for GT6, which has no additional 5′ ATG residues. Thus, hypothesis ii is an unlikely explanation of the above observations.

Under hypothesis iii, upstream AUGs are removed by splicing. Since PB165 and PB168 have similar RNA sequences except for the number of *Cla*I linkers, the 10-fold difference in their TKTU appears inconsistent with this hypothesis. In addition, the presence of cryptic splicing sites would result in the loss of sequences and would thereby generate smaller viral genomes (18, 19). However, smaller viral genomes were not detected when chicken embryo fibroblasts were infected with PB101, PB160, and GT2. Thus, such cryptic splicing does not appear to be a frequent event during viral infection.

It appears, therefore, that in PB158, PB168, PB165, and PB160 there is initiation at the normal initiator codon of the *tk* gene. This initiation occurs even though there are 5′ AUG codons, including some which are present in the canonical sequences, one in PB158 and seven in PB160.

However, the level of TK as reflected in the specific TKTU is influenced by upstream sequences. For example, PB165 is 1/10 as active as PB158 and PB168. These constructs differ in the number of ATGs present due to the insertion of *Cla*I linkers. The ATG residues in *Cla*I linkers are not present in the consensus sequence and hence may not serve as signals for initiation of protein synthesis. In such a case the *Cla*I linkers merely serve as additional 5′ sequences which serve to modulate the expression of the *tk* gene.

These experiments demonstrate that there can be efficient initiation at internal codons, but that the efficiency of this initiation is affected by the nucleotide sequence 5′ to the internal initiation codon.

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