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Expression of Complete Chicken Thymidine Kinase Gene Inserted in a Retrovirus Vector

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The chicken thymidine kinase (*tk*) gene was inserted into spleen necrosis virus. Thymidine kinase activity was expressed even when the promoter and terminator sequences for *tk* RNA synthesis were retained. When the promoter was present in the same orientation as the promoter in the long terminal repeat of the virus, deletions occurred both in the virus and in the *tk* gene, and the thymidine kinase-transforming activity of the recovered virus was low. Splicing of apparent intervening sequences in the *tk* gene was also observed. When the orientation of the *tk* promoter was opposite to the promoter in the long terminal repeat, virus synthesis was diminished, whereas thymidine kinase activity was expressed at an elevated level compared with virus in which the promoter was in the same orientation. However, when the apparent *tk* promoter was deleted from virus with the *tk* gene in the opposite orientation, a high level of virus synthesis was observed, probably as a result of absence of interference of RNA synthesis from converging promoters. The intervening sequences in the virus in which the promoters were in opposite orientation were not spliced.

Retroviruses are single-stranded RNA viruses that replicate through an obligatory DNA intermediate (2, 16). This DNA intermediate can integrate into the host genome and be propagated as a cellular genetic element. This property has made retroviruses useful for the introduction of exogenous genes in vertebrate cells in a stable manner (8, 11, 15, 17). When the coding sequences of an exogenous gene are inserted in a retrovirus vector, the initiation and termination signals for the synthesis of the mRNA of the inserted gene can be provided by the exogenous gene or by the same nucleic acid sequences that the virus utilizes for its replication. For example, Shimotohno and Temin (11) previously studied the expression of thymidine kinase (TK) activity in a recombinant retrovirus containing the herpes simplex virus (HSV) thymidine kinase (*tk*) gene containing its own promoter and termination signals. It was observed that it was necessary to remove the polyadenylate addition signal from the *tk* mRNA for the efficient production of TK-transforming virus. We have also studied this *tk* gene without its promoter (1).

To study the expression of a cellular gene carrying its own promoter and termination signals, the chicken *tk* (ch *tk*) gene was introduced into a retrovirus vector. The ch *tk* gene was isolated by Perucho et al. (9) as a *Hin*dIII fragment of three kilobase pairs (kbp). The active gene is contained in a 2.3-kbp *Eco*RI-*Hin*dIII DNA fragment which has both the promoter and termination signals for the synthesis of *tk* mRNA. The *tk* mRNA is about two kilobases and polyadenylated. The gene has at least three small introns (6).

We studied the expression of TK activity when the *tk* gene was inserted into a retrovirus vector constructed by using spleen necrosis virus (SNV). The *tk* promoter was present in the same orientation as the promoter in the 5′ long terminal repeat (LTR) of the virus in the construct pPB103 and in the opposite orientation in pPB104. In the experiments described in this paper, the initiation and termination of *tk* mRNA synthesis may occur at sites present in the virus for virus production. However, the synthesis of *tk* mRNA may also be initiated at the transcription signals endogenous to the *tk* gene. Also, proper splicing of the intervening sequences in the *tk* gene is necessary for the expression of TK activity.

We studied virus synthesis and *tk* expression in pPB103 and pPB104. We observed that when the *tk* promoter is in the same orientation as the 5′ LTR, virus production is efficient, whereas *tk* expression is inefficient. The reverse is the case when the promoters are in opposite orientation, that is, virus production is inefficient, whereas *tk* expression is efficient. Loss of the intervening sequences of the *tk* gene and deletion of viral sequences was also observed, especially when both virus and cell promoters had the same orientation.

MATERIALS AND METHODS

Cells, methods for transfection and infection of cells, selection of transformants, and isolation and characterization of unintegrated viral DNA have been previously described (1).

Construction of SNV-ch *tk* recombinant plasmids. Plasmid pchtk-5 (9) was obtained from M. Wigler. It contains the ch *tk* gene in a 3-kbp *Hin*dIII fragment, inserted at the *Hin*dIII site of pBR322. The isolated gene has its own promoter and termination signals for RNA synthesis (6).

Plasmid pPB101, a complete DNA clone of SNV in pBR322, was digested with *Bgl*II. The resulting single-stranded termini were repaired by treatment of the digested DNA with the Klenow fragment of *Escherichia coli* DNA polymerase I. The DNA was ligated to *Hin*dIII linkers, digested with *Hin*dIII, and ligated to form pPB102.

Plasmid pchtk-5 (Fig. 1) was digested with *Hin*dIII and electrophoresed in a 0.8% agarose gel. The fragment containing the *tk* gene was isolated by using glass powder (W. Studier, personal communication). pPB102 was digested with *Hin*dIII and ligated to the isolated *Hin*dIII fragment containing the *tk* gene. The ligated DNA was used to transform *E. coli* HB101 cells. The ampicillin-resistant colonies were screened for the presence of a 3-kbp insert at the *Hin*dIII site in pPB102. The plasmid DNAs from these

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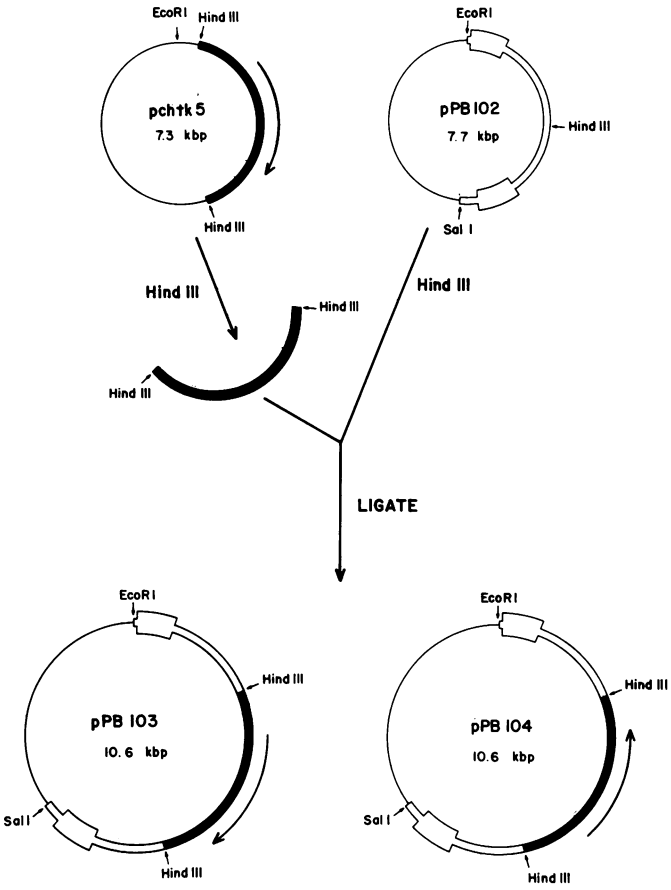

FIG. 1. Construction of pPB103 and pPB104. Open bar is SNV sequence; large open box is LTR; single line is pBR322; dark segment is the ch *tk* gene. The arrows adjacent to the ch *tk* gene show the direction of transcription from the *tk* promoter.

colonies were digested with *Kpn*I or *Eco*RI to determine the orientation of the *tk* insert in pPB102. In pPB103, the *tk* gene is present in the same orientation as SNV, whereas in pPB104 it is in the opposite orientation (Fig. 1).

Plasmid pPB104 was digested with *Sma*I or *Bgl*II. Thirty micrograms of this DNA was digested with 2 U of BAL-31 nuclease at 37°C. Samples of the reaction mixture were removed at 30, 60, 90, and 120 s; diethyl pyrocarbonate was added to a final concentration of 0.2%; and the mixture was incubated at 37°C for 15 min. The DNA was then ligated and used to transform HB101 cells. The plasmid DNAs from ampicillin-resistant colonies were digested with a combination of restriction endonucleases to determine the size of the deletions produced after digestion with BAL-31 nuclease (see Fig. 4).

Cloning of unintegrated viral DNA from chicken embryo fibroblasts infected with PB103 was done according to procedures described by Shimotohno and Temin (12).

Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. The enzyme digestions were carried out according to conditions described by the suppliers. *Hin*dIII linkers, CGCAAGCTTGCG, were purchased from Collaborative Research, Inc., Waltham, Mass.

RESULTS

We inserted the ch *tk* gene in SNV DNA to produce recombinant plasmids pPB103 and pPB104. pPB103 is an SNV-ch *tk* recombinant plasmid in which the direction of transcription of the *tk* gene from its promoter is the same as the direction of transcription of viral RNA from the promoter in the LTR. pPB104 is a recombinant in which the *tk* gene promoter is in the opposite orientation to that in the LTR.

Expression of TK activity by recombinant plasmids. When DNAs from pPB103, pPB104, and pchtk-5 were used to transfect LMTK$^-$ cells, the efficiencies of transformation to the TK$^+$ phenotype were comparable (Table 1). However, the virus recovered from chicken embryo fibroblasts transfected with pPB103 had a much lower transforming activity than virus recovered from transfection with pPB104 (Table 1).

To determine whether the lower TK-transforming units (TKTU) titer of PB103 virus was a result of lower virus production, we determined the amounts of unintegrated viral DNA synthesized in chicken embryo fibroblasts infected with virus harvested from chicken embryo fibroblasts transfected with pPB103 or pPB104 along with a plasmid containing helper virus DNA, reticuloendotheliosis virus strain A (REV-A). Figure 2A shows that cells infected with virus that was recovered after transfection with pPB104 contained reduced amounts of unintegrated viral DNA compared with cells infected with virus that was recovered from pPB103. The helper REV-A DNA was present in comparable amounts in both samples (Fig. 2B). Thus, although pPB104 has a lower efficiency of virus production than pPB103, the virus produced is at least 1,000 times as efficient in conferring the TK$^+$ phenotype to TK$^-$ cells (Table 1).

Structure of unintegrated viral DNA of PB103 and PB104. To determine the reason for this difference in specific TKTU, we determined the structure of unintegrated viral DNA in cells infected with virus recovered from pPB103 and pPB104. The DNA recovered from cells infected with PB103 virus was smaller than that recovered from cells infected with PB104 virus, although pPB103 and pPB104 are the same size (Fig. 2).

To determine when PB103 virus became smaller, we examined the unintegrated viral DNAs on successive days after transfection of chicken embryo fibroblasts and observed that viral DNA was smaller by the second day, the earliest time at which we could detect unintegrated viral DNA (data not shown). The size of the PB103 DNA was identical on all days.

To determine the location of the deletion in PB103 virus,

TABLE 1. Biological activity of SNV-ch *tk* recombinants

Clone	TKTU/μg of DNA[a]	Relative TKTU of virus recovered[b]	Relative yield of unintegrated virus DNA (SNV-ch *tk* DNA/REV-A DNA)[c]
pPB103	100	1	0.4
pPB104	150	170	0.05
pchtk-5	170		

[a] LMTK$^-$ cells were transfected with DNA as indicated, and the number of TK$^+$ transformants was determined (see the text). Values represent an average of three experiments.

[b] Virus recovered 5 days after transfection of chicken embryo fibroblasts with plasmid DNA was harvested and assayed on ch *tk*$^-$ cells. The absolute values of TKTU per milliliter varied from experiment to experiment, but the relative TKTU of PB104 to PB103 was always between 1×10^2 and 3×10^2/ml. The TKTU of virus recovered from pPB104 was between 10^4 and 10^5/ml.

[c] Values were obtained from Fig. 2.

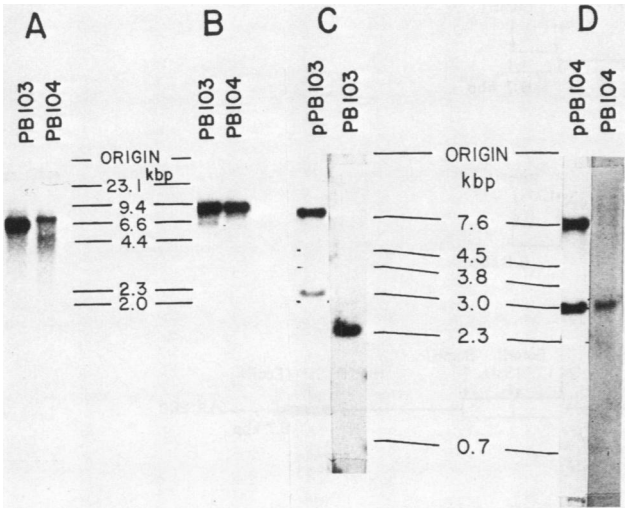

FIG. 2. Southern blot of unintegrated viral DNA isolated from chicken embryo fibroblasts infected with virus harvested from chicken embryo fibroblasts previously transfected with pPB103 or pPB104 in the presence of DNA containing REV-A as helper. (A) Nitrocellulose paper was hybridized to nick-translated ^{32}P-chtk-5 DNA. (B) Nitrocellulose paper used in (A) was rehybridized to nick-translated ^{32}P-SNV DNA. (C) *Hin*dIII digest of pPB103 and of unintegrated viral DNA obtained from cells infected with pPB103 hybridized to nick-translated ^{32}P-chtk-5 DNA. (D) *Hin*dIII digest of pPB104 and of unintegrated viral DNA obtained from cells infected with PB104 hybridized to nick-translated ^{32}P-chtk-5 DNA. The 7.6-kbp band in panels C and D in the lanes corresponding to pPB103 and pPB104 represents the fragment containing pBR322 and SNV DNAs.

unintegrated viral DNA was digested with *Hin*dIII and analyzed (Fig. 2C). The nucleotide sequences deleted from PB103 appear to be in the region of the ch *tk* gene. The fragment of DNA containing *tk* DNA is 2.3 kbp in PB103, whereas it is 3.0 kbp in PB104 (Fig. 2C and D).

The unintegrated viral DNA, PB103, that was recovered from pPB103 was mapped by restriction enzyme cleavages (Fig. 3b). The map represents the structure of the major species of *tk* DNA-containing molecules present. (The major species of DNA molecule present is determined by the rate of replication of the virus, not by the efficiency of *tk* expression.) SNV DNA 5′ and 3′ to the ch *tk* sequences was maintained unaltered, and internal regions of the *tk* gene were deleted.

To determine the structure more precisely, DNA clones were made of virus recovered after transfection with pPB103. Closed circular viral DNA recovered from cells infected with PB103 was cloned in λ phage Charon 21A at the unique *Eco*RI site. The structure of such a cloned virus, λPB103B, is shown in Fig. 3d. It has only one LTR and is deleted both in the 5′ SNV and the *tk* sequences. Three other independent isolates of recombinant phage have the same structure (data not shown). The deletions in the *tk* region appear to be the same as those observed in the population of total unintegrated viral DNA, PB103, shown in Fig. 3b.

Analysis of virus from transformed TK$^+$ cell clones. Some virus harvested from chicken embryo fibroblasts after transfection with pPB103 is active in transforming ch *tk*$^-$ cells to the TK$^+$ phenotype (Table 1). We isolated some clones of ch TK$^+$ cells. The viruses obtained from five such clones were tested for their ability to transform buffalo rat liver TK$^-$ cells to the TK$^+$ phenotype. The virus produced by three of these TK$^+$ clones had no TK-transforming activity, and the virus from two others had high titers of TK-transforming activity (10^5 TKTU/ml) (data not shown).

We analyzed the unintegrated viral DNA synthesized by chicken embryo fibroblasts infected with virus recovered from one such productive clone, B6 (Fig. 3e). The DNA is small in comparison with the virus expected from pPB103 (Fig. 3a). The SNV DNA 3′ to the chicken region appears unaltered. However, internal regions in the ch *tk* fragment have been deleted, and the 5′ region of the *tk* gene, including the promoter and some adjoining SNV DNA, has been deleted.

Clones of ch TK$^+$ cells were also isolated from ch *tk*$^-$ cells infected with virus obtained from pPB104. Three such clones were tested for production of TK-transforming activity. Virus obtained from one of these clones produced transforming activity, whereas virus from the other two did not. We have not analyzed these viruses further except to show that the unintegrated viral DNA synthesized after infection with virus from the one productive clone was 7.2 and 8.5 kbp, larger than expected from pPB104 (data not shown).

Effect of *tk* "promoter" deletion in pPB104. Since the direction of transcription from the promoter of the ch *tk* gene in pPB104 is opposite to that from the viral LTR promoter, it seemed possible that the synthesis of reduced amounts of virus in cells infected with PB104 virus was a result of interference with virus RNA synthesis. To test this hypothesis, we constructed various plasmids from pPB104 by deleting with BAL-31 nuclease in the region of DNA around the unique *Sma*I site (Fig. 4). The initiation of transcription of ch *tk* mRNA is thought to be close to the *Kpn*I site adjacent to the *Sma*I site (6). The relative amounts of unintegrated viral DNA produced in cells infected with virus derived from the various deleted strains of pPB104 are shown in Fig. 4. When the deletion included the apparent promoter, there was enhanced synthesis of unintegrated viral DNA.

DISCUSSION

Expression of *tk* gene inserted with promoter and polyadenylate addition signals for RNA synthesis. To study the expression of a cellular gene from internal promoters, we constructed recombinant retroviruses in which the ch *tk* gene with its promoter was inserted into SNV DNA. The *tk* promoter was present either in the same (pPB103) or opposite (pPB104) orientation compared with the promoter in the LTR.

In the experiments presented in this paper, TK activity was not measured directly but rather is reflected in TKTU, which measure the ability of *tk*-containing virus to transform TK$^-$ cells to the TK$^+$ phenotype. Spandidos and Wilkie (14) observed that the ability of recombinant plasmids to transform TK$^-$ recipient cells to the TK$^+$ phenotype was reflected in their ability to synthesize *tk*-specific RNA and enzyme in recipient cells. Santangelo and Cole (10) also reported a direct correlation between the level of transient expression of the HSV *tk* gene and the frequency of biochemical transformation of cells to the TK$^+$ phenotype.

Although the TK-transforming activities of both the plasmids were comparable, the specific TKTU were considerably different when the corresponding viruses were used. In addition, unintegrated viral DNA recovered from pPB103 was smaller than that from pPB104.

Promoter in same orientation (pPB103). The ch *tk* gene has

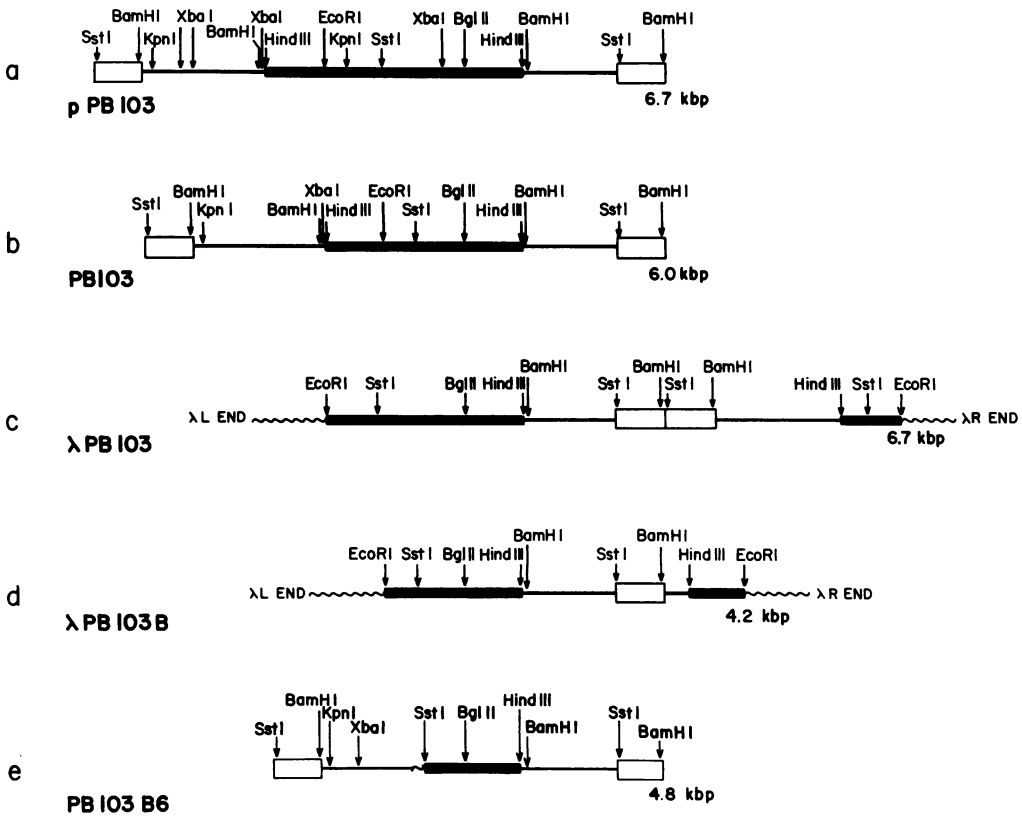

FIG. 3. Structure of unintegrated viral DNAs synthesized by chicken embryo fibroblasts infected with virus recovered from chicken embryo fibroblasts transfected with pPB103 in the presence of REV-A DNA as helper. (a) Structure of viral DNA in pPB103. The open boxes represent LTRs; the single line, DNA from SNV. The dark box is ch *tk* DNA. Sizes refer to the SNV-ch *tk* insert. (b) Restriction enzyme cleavage site map of total unintegrated viral DNA, PB103. (c) Expected structure of circular unintegrated PB103 DNA inserted at the *Eco*RI site in Charon 21A. The wavy line represents DNA from Charon 21A. (d) Restriction map of a molecular clone, λPB103B, of recovered circular unintegrated viral DNA inserted at the *Eco*RI site of Charon 21A. (e) Structure of virus DNA from the ch TK^+ clone (PB103B6). ~ indicates uncertainty of the junction between SNV and ch *tk* DNA.

multiple intervening sequences (6). The smaller size of the unintegrated PB103 DNA is probably the result of splicing of intervening sequences from the recombinant SNV-ch *tk* RNA. Such splicing has been previously observed in genes inserted in retrovirus vectors (12, 13).

Cloned virus, either from a DNA clone (PB103B) or from a cell clone PB103B6, was even smaller. Deletions are common in retrovirus replication, and unintegrated circular virus DNA with one or two LTRs is synthesized (16). Hence, it is common during cloning of circular unintegrated DNA to obtain species that are deleted or have only one LTR. In PB103B, 5′ viral sequences are deleted, whereas the apparently spliced *tk* gene and 3′ viral sequences are maintained. In PB103B6, the *tk* gene is also apparently spliced, and the *tk* promoter and 5′ sequences are deleted, whereas the 3′ viral sequences are maintained.

Deletion of the promoter of an inserted gene is probably a general phenomenon. Most naturally occurring retroviruses do not contain internal promoters. The modified virus from clone B6 (PB103B6) may have been selected because the absence of the additional promoter confers stability to the virus. Similar deletions have also been observed by Emerman and Temin (3). They observed that when cells were infected with SNV-α-globin-*tk* recombinant viruses in which a mouse α-*globin* gene with its promoter was inserted 5′ to the HSV *tk* gene, the recovered viruses had a high efficiency of TK-transforming activity and were deleted. The deletion led to the loss of the globin promoter. When the initial construct lacked the sequence for the globin promoter, such deletions did not occur.

The presence of polyadenylate addition signal within the *tk* sequences does not inhibit virus production by pPB103. These sequences, which are present about 20 bases upstream from the 3′ *Hin*dIII site in the *tk* gene, may also be present in the recovered virus unless a small deletion or mutations occurred at this site which remained undetected in the restriction enzyme analysis (Fig. 3). However, Shimotohno and Temin (11) observed that it was necessary to remove the polyadenylate addition signal from the HSV *tk* gene for the efficient production of TK-transforming virus.

The specific TKTU of PB103 virus is 1/1,000 that of PB104. We observed that the ch *tk* gene is apparently spliced in the recovered virus. If the *tk* promoter is used for the synthesis of *tk* mRNA, little functional mRNA may be made due to the *tk* sequences in the provirus being already processed by splicing of the virus RNA. Hamer and Leder (4) constructed a series of simian virus 40 transducing viruses carrying various combinations of splice junctions derived from the viral genome and a mouse globin gene. They observed that virus from which all the splice junctions were removed failed to produce any detectable stable RNA. Using similar recombinant viruses containing rabbit β-globin cDNA, Hamer et al. observed that presence of viral intervening sequences was necessary for the formation of a stable

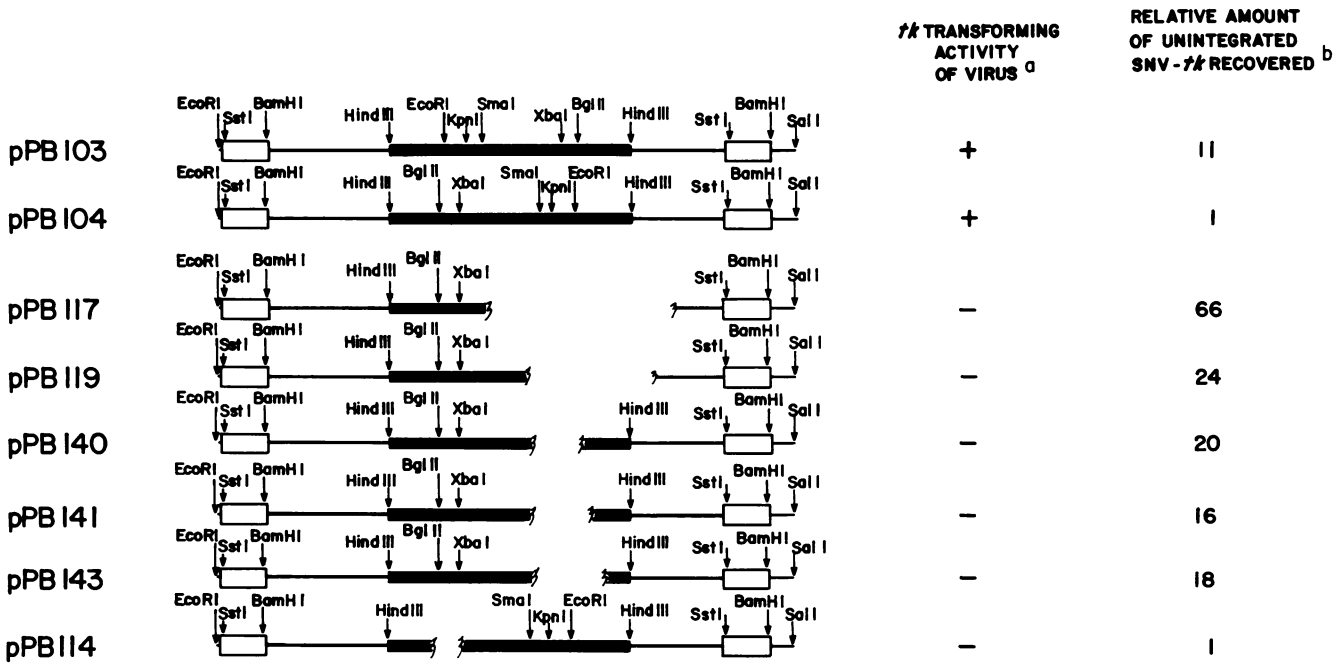

FIG. 4. Effect of deletions in the *tk* gene in pPB104 on the recovery of virus. Deletions in the *tk* gene in pPB104 were constructed as described in the text. Structures of the insert in pPB104 and deleted plasmids are schematically represented. pPB103 has been included for comparison. The open box represents an LTR; the single line, SNV DNA. The dark box is ch *tk* DNA. The interruptions in the constructs represent approximate extents of deletions. ⟆⟆ indicates uncertainty in the location of the boundaries of the deletions. Recognition sites of various restriction endonucleases are shown. [a]Negative TK-transforming activity indicates TKTU less than 0.1% of the virus considered positive for TK-transforming activity. [b]Chicken embryo fibroblasts were transfected with the plasmid in the presence of plasmid containing REV-A DNA as helper. Virus harvested 5 days after transfection was used to infect chicken embryo fibroblasts, and unintegrated viral DNA was isolated 3 days after infection. The amounts of SNV-ch *tk* and REV-A DNA were quantified. Results are presented as relative amounts with SNV-ch *tk* DNA/REV-A DNA from pPB104 as 1.0.

simian virus 40-globin hybrid transcript (5). Alternatively, there may be reduced synthesis of *tk* mRNA due to interference between the viral and cellular promoters. However, such interference is not seen during expression of the HSV *tk* gene (11) in the presence of both the viral and *tk* promoters. Another possibility is that the enzyme is synthesized from full-length viral RNA. In this case, TK synthesis would have to initiate at an internal AUG codon, resulting in reduced TK activity (1).

Promoter in opposite orientation (PB104). The unintegrated viral DNA recovered after infection of chicken embryo fibroblasts with PB104 virus was of the expected size. In PB104 the strand complementary to the viral RNA codes for the *tk* mRNA. Thus, the viral RNA does not have any of the splicing signals in the region of the *tk* gene and is not processed to a smaller size, as in the case of PB103.

Virus harvested from transfection of chicken embryo fibroblasts with PB104 has high specific TK-transforming activity in spite of the low yield of virus. Even though virus synthesis is depressed by the presence of the opposing *tk* promoter, there must be an abundance of *tk* mRNA. Perhaps the *tk* promoter is stronger than the viral promoter. Interference with gene expression as a result of convergent transcription is known to occur in procaryotes (7). By using λ *trp* transducing phages, it was shown that gene expression directed by both promoters *trp* and λ p_L was mutually impaired, with that from the *trp* promoter being totally blocked. Expression from the *trp* promoter was, however, restored when p_L was turned off.

Higher yields of virus were obtained from viruses in which the apparent promoter for the *tk* gene oriented in the opposite direction was deleted. This increase probably reflects increased production of virus as a result of the absence of interference by the opposing promoter. However, in the absence of nucleotide sequence information, it cannot be ruled out that the deletions removed fortuitous termination signals for RNA synthesis in the minus strand of the *tk* gene.

It appears plausible that if a promoter or other positive controlling element in a gene is "appropriately attenuated," the gene may be inserted in a retrovirus vector in the opposite orientation, resulting in production of virus while allowing expression of the inserted gene. The lower rate of transcription from such a defective promoter would be compensated by the availability of large numbers of templates due to increased synthesis of virus. No splicing or deletions in the inserted gene would be expected to occur.

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