Inhibition of the herpes simplex virus thymidine kinase gene transfection in *Ltk*⁻ cells by potential Z-DNA forming polymers

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ABSTRACT

It has been demonstrated that certain alternating purine and pyrimidine sequences may assume a left-handed Z-DNA conformation. In order to evaluate the possibility that Z-DNA is involved in the modulation of gene expression, we examined the ability of various synthetic DNA polymers to affect the transfection of herpes simplex virus thymidine kinase (HSVtk) gene in *Ltk*⁻ cells using the DNA-calcium phosphate cotransfection technique. We found that potential Z-DNA forming polymers such as, poly(dG-m⁵dC).poly(dG-m⁵dC) and poly(dG-dC).poly(dG-dC), cotransfected with the tk gene decreased the level of Tk⁺ transformed colonies. In contrast, cotransfection of the tk gene with polymers which do not assume Z-conformation such as, poly(dG).poly(dC) or poly(dA-dT).poly(dA-dT) showed no effect on the number of colonies formed. About 50% inhibition of the Tk⁺ colony formation was obtained by 0.4 µg of poly(dG-m⁵dC).poly(dG-m⁵dC), or by 2 µg of poly(dG-dC).poly(dG-dC). DNA uptake into *Ltk*⁻ cells was not significantly affected by any of these polymers. Approximately 20-42 base pairs (bp) long alternating dG-dC sequence linked at either the 5'-end or 3'-end of tk gene were cloned into plasmids. These recombinant plasmids, however, showed no remarkable effect upon the transfection of *Ltk*⁻ cells. The DNAs of Tk⁺ colonies obtained by transfecting these recombinant plasmids were digested with BssH II and analyzed by Southern blotting. We demonstrated that the dG-dC sequences proximal to the tk gene were integrated into cellular DNA. All the presented results indicate that only larger polymers with the potential to assume a Z-DNA conformation may affect tk gene transfection either by inhibiting transcription or more probably by affecting the stable integration of the tk gene into the host chromosome.

INTRODUCTION

Recent studies have demonstrated the existence of a left-handed Z conformation in synthetic DNA polymers containing certain alternating purine-pyrimidine sequences (1-7). Left-handed helices have also been detected in segments of DNA restriction fragments and recombinant plasmids (3,8-10). Various types of Z-DNAs have been used as immunogens to specific antibodies that recognize Z-DNA conformations (11-14). These antibodies can detect regions of Z-forming potential in naturally occurring DNAs including the chromosomes of various species (15-21).

Despite information about the structure and distribution of Z-DNA sequences, little is known about their function in biological systems (22). It was recently proposed that Z-DNA may play a role in gene expression (8). Nordheim et al. (23) suggested that Z-DNA may act as a positive regulatory element stimulating the enhancer sequence of SV-40 DNA. Increased transient expression of the chloramphenicol acetyl-transferase (CAT) gene was observed when a 30-130 bp long (dT-dG).(dC-dA) sequence was inserted into the CAT expression vector plasmid (24). Conversely, it has been shown that Z-DNA may also play a role as a negative controlling element in the transcription of a tRNA gene by polymerase III in vitro (25,26). Additionally, it has been demonstrated that DNA in the Z-conformation is a considerably less active template than one in the B conformation when transcribed by E. coli RNA polymerase (27) or by wheat germ polymerase II (28). Recently, using a plasmid containing a (CG)₁₆ sequence, it has been shown that a transcriptional blockage occurs when the template is converted from B to the left-handed Z-form (29).

Since herpes simplex virus thymidine kinase (HSVtk) is an excellent system to evaluate the efficiency of exogenous gene transfection (30,31), we have examined the effect of Z-DNA on Tk⁺ colony formation by cotransfecting the HSVtk gene with various potential Z-DNA forming polymers in Ltk⁻ cells. We also constructed recombinant plasmids containing approximately 20-42 bp tracts of alternating (dG-dC) sequences at the 5' and 3' ends of tk gene and used them for transfection assay. In both of these approaches, we scored for Tk⁺ transformants by selection in hypoxanthine-aminopterin-thymidine (HAT) medium.

MATERIALS AND METHODS

Materials

Plasmid ptk5 and Ltk⁻ cells were obtained from Dr. R. Axel, and pSV2 gpt from Dr. L. Chasin. Other materials were purchased from the following companies: restriction enzymes and T4 DNA ligase, New England Biolabs; DNA polymerase I and calf intestinal alkaline phosphatase, Boehringer Mannheim; [³²P]-labeled nucleotides, Amersham; Biogel A-15m agarose beads, Bio-Rad Corporation; all synthetic polynucleotides, Pharmacia-PL Biochemicals; nitrocellulose filter (BA85), Schleicher and Schuell; Kodak AR 10 X-ray film, Picker Corp; cell and bacterial media from Gibco and Difco, respectively.

Methods

Cells and the transfection protocol

Mouse Ltk⁻ cells were routinely maintained in Dulbecco's modified Eagle Medium supplemented with 10% calf serum (v/v) and kept in a humidified incubator at 37°C with 5% CO₂. Cell transformation was performed by the calcium phosphate precipitation method (32) as modified (31) by Wigler et al. Ltk⁻ cells (6 x 10⁵) were seeded into a 90 mm dish, grown for 24 hours. They were fed with fresh medium 4 hours before transfection with the DNA-calcium phosphate precipitate. The precipitate was formed with 10 µg of Ltk⁻ high molecular weight carrier DNA and 0.2 to 1.0 µg of tk gene-containing plasmid and polynucleotides as indicated. After 4 hours of exposure to the DNA precipitate, the medium was replaced and incubation continued for an additional 24 hours. At this point, cells were exposed to selective HAT medium containing 15 µg/ml hypoxanthine, 0.2 µg/ml aminopterin and 5.0 µg/ml thymidine. The medium was changed the next day and then every 2-3 days until Tk⁺ colonies developed (2-3 weeks). The colonies were either scored following staining with giemsa or isolated and expanded for further analysis.

In some of the experiments with the dG-dC inserted recombinant plasmids, we used a double selection procedure. First, we selected for Gpt⁺ colonies in a growth medium containing 15 µg/ml hypoxanthine, 2.5 µg/ml xanthine, and 2.5 µg/ml mycophenolic acid, and then we subjected these Gpt⁺ colonies to HAT selection in order to obtain Tk⁺ colonies. Only colonies displaying both positive phenotypes were isolated and analyzed by Southern blotting.

DNA preparations and plasmids

Cellular DNAs were purified by a described procedure (33). Plasmid DNAs were purified by gel filtration through Biogel A-15m agarose beads. The plasmids ptk5 contains a 3.6 kb BamH I fragment of Herpes simplex virus type 1 (HSV-1) DNA cloned in the plasmid, pBR322. The BamH I fragment contains a 2.02 kb Pvu II fragment comprising the 1.3 kb tk gene coding region (34,35,36). The Pvu II fragment was fractionated by electrophoresis in a 0.7% agarose gel and purified by electroelution.

Construction of plasmid containing a tract of alternating dG-dC sequences linked to the tk gene

The plasmid pSV2gpt containing the E. coli gpt gene has been previously described (37,38). The Pvu II fragment containing the tk gene was cloned into the Pvu II site on pSV2gpt plasmid DNA. A Tk⁺, Gpt⁺ recom-

Figure 1. Construction of plasmids containing tracts of alternating dG-dC sequences. The derivation of p33tk and the three steps involved in formation of the dG-dC carrying constructs is described in Materials and Methods. The plasmid, **tk⁺**, and **gpt⁺** DNA segments have been drawn to scale. The sizes of the dG-dC and SV40 origin segments have been exaggerated for the sake of clarity. The **tk** gene is represented by a segment of hatched marks. The **gpt** gene including the polyadenylation signal derived from SV40 is represented by a filled-in bar segment. The dG-dC inserts on plasmid p30Ztk and p27Ztk are represented by a zig-zag line. The SV40 origin of replication is shown by an open bar segment.

binant plasmid was isolated and designated p33tk. The scheme for cloning a segment of dG-dC sequence proximal to the **tk** gene insert on p33tk is outlined in Figure 1.

In the first step of the construction, plasmid p33tk was partially cleaved with **Pvu II**. We established that 0.1 units of enzyme per 1 μ g of DNA at 37°C for 90 min gave a maximum yield of linearized plasmid. The linearized DNA was twice purified by preparative electrophoresis and electroelution. Step 2 shows the mixed digestion products. Linearized p33tk DNA was next reacted with phosphatase as described by Maniatis *et al.* (39). In step 3, alternating dG-dC polymer was digested with **FnuD II**. Fragments were fractionated by electrophoresis and pieces ranging in size from 200-300 bp were isolated by electroelution. The purified polymer fragments were ligated to the linearized p33tk DNA. Digestion and ligation

conditions were as specified by the manufacturer. Recombinant colonies carrying dG-dC inserts were screened by the Hogness-Grunstein method (40) using nick-translated (41) 200-300 bp FnuD II dG-dC fragments as a probe. Positive clones were cleaved with BssH II to demonstrate the orientation of the dG-dC segment with respect to the tk gene. Next, the DNAs of clones in which the insert was 5' of tk were cleaved with Eco RI and Tth III, and those in which the insert was 3' of tk were cut with Sma I and Bgl I. Plasmid p33tk was similarly digested for comparison. The DNA samples were electrophoresed in a 4% polyacrylamide gel in order to determine the approximate lengths of the dG-dC stretches. Plasmids designated p30Ztk and p31Ztk contained inserts 5' of tk of about 40 bp and 20 bp, respectively. Another plasmid designated p27Ztk contained an insert 3' of tk of about 42 bp.

Southern blot hybridization

Genomic DNAs from the clones isolated by double selection were cleaved with the restriction enzyme BssH II. The digested DNAs were electrophoresed in a 0.7% agarose gel, and transferred to nitrocellulose filters by the Southern blotting technique (42). Hybridization of the nick-translated (41) tk Pvu II fragment to the filter-bound genomic DNAs was performed as described (43).

DNA uptake analysis

A modified method of Loyter *et al.* (44) was used to determine DNA uptake into the Ltk⁻ cells co-transfected with various synthetic polymers. Briefly, the cells were transfected with 10 µg of Ltk⁻ carrier DNA combined with the nick-translated [³²P]-labeled tk Pvu II fragment containing about 6 x 10⁵ cpm and various polymers. About 48 hours later, the cells were washed three times with 10 ml 4°C Ca/Mg-free phosphate-buffered saline (PBS), followed by two washings in 1 x PBS containing 5 mM of both EGTA and EDTA. The cells were then lysed by addition of a buffer (1 ml/plate) containing 10 mM Tris HCl (pH 7.9), 10 mM EDTA, and 0.5% sodium dodecyl sulfate. The cellular DNA was precipitated with 20% (wt/vol) trichloroacetic acid (TCA) containing 10 mM pyrophosphate and 0.25% tungstic acid, and applied to Whatman GF filters. After air drying, the filters were counted in a liquid scintillation counter to determine the amount of TCA-precipitable [³²P]-DNA incorporated into the cells. In some experiments, nuclei and cytoplasmic fractions were separately isolated from the transfected cells upon treatment with triton X-100 followed by TCA precipitation and counting.

RESULTS

Effect of polynucleotides on the efficiency of tk gene transfection

Since the biological role of Z-DNA conformation is not well understood, we have designed experiments that address the possible role of the Z-DNA in modulating gene expression by evaluating the total number of transfected Tk^+ colonies formed in HAT medium. We have used a variety of polynucleotides, with and without the potential to assume Z-conformation, in a cotransfection assay system. It has been observed that using the calcium phosphate precipitation method exogenously acquired sequences such as the HSVtk gene and carrier DNA tend to become physically linked upon entering the cell nuclei and are often randomly integrated into the host chromosomes (45). We have used this technique to determine the effect of different synthetic polynucleotides on tk gene transfection in Ltk^- cells.

To evaluate the efficiency of tk cotransfection with two potential Z-DNA polymers, poly(dG-m⁵dC).poly(dG-m⁵dC) and poly(dG-dC).poly(dG-dC), we used two forms of plasmid ptk5 DNA: circular and linear for transfection of Ltk^- cells. The ptk5 DNA was linearized by cleavage at the single Hind III site. This enzyme cuts in plasmid DNA sequences near the 5' end of tk gene. The use of linearized plasmid DNA appears to increase transfectability (46) by providing a substrate for the end-to-end ligations that precede integration (47). We also used the purified 2.02 kb Pvu II fragment of plasmid that contains the 1.3 kb coding region of the tk gene in cotransfection experiments.

We first determined the effect of two potential Z-DNA forming polymers on the tk gene by cotransfecting with the purified Pvu II fragment. Figure 2 shows the effect of different concentrations of the methylated and unmethylated polymers on cotransfection of Ltk^- cells using this Pvu II fragment. Poly(dG-m⁵dC).poly(dG-m⁵dC) is more potent than poly(dG-dC).poly(dG-dC). Only 0.4 μ g of this methylated polymer is required to achieve 50% inhibition, whereas 2.0 μ g of poly(dG-dC).poly(dG-dC) is required to induce this level of inhibition. Then we performed similar cotransfection experiments (Table I), using the circular and Hind III-linearized forms of ptk5 plasmid, which also demonstrated that poly(dG-m⁵dC).poly(dG-m⁵dC) is more potent at inducing 50% inhibition of Tk^+ colony formation than the poly(dG-dC).poly(dG-dC). Thus, the methylated polymer which has a greater tendency to be in the Z-conformation also exerts the strongest inhibition on tk gene expression using any of the three forms of tk DNA. We have also compared the efficiency of gene transfection of other

Figure 2. Effect of poly(dG-m⁵dC).poly(dG-m⁵dC) and poly(dG-dC).poly(dG-dC) on cotransfection with the Pvu II fragment of plasmid ptk5. (—) poly(dG-m⁵dC).poly(dG-m⁵dC), (----) poly(dG-dC).poly(dG-dC).

polymers with the potential to form Z-DNA, such as poly(dG-dC).poly(dG-dC)-AAF and poly(dA-dC).poly(dG-dT) or polymers which do not assume Z-conformation, such as poly(dG).poly(dC) and poly(dA-dT).poly(dA-dT). In these experiments, we have used a high concentration of polymers (10 μg) which in the cases of poly(dG-m⁵dC).poly(dG-m⁵dC) or poly(dG-dC).poly(dG-dC) are completely inhibitory. The poly(dG-dC).poly(dG-dC) modified by AAF

Table I. Amount of polynucleotides required to induce 50% inhibition of Tk⁺ colonies by cotransfection with tk⁺ gene

ptk5 (μg)	Poly(dG-dC) (μg)	Poly(dG-m ⁵ dC) (μg)
Circular (1 μg)	8.1	2.3
Linearized (1 μg)	4.6	1.5
Pvu II fragment (0.2 μg)	2.0	0.4

10 μg of Ltk⁺ carrier DNA was used for transfecting each plate. Average number of colonies from four different experiments, by using circular, linearized and Pvu II fragment were 336, 390, and 470, respectively.

Table II. Effect of polynucleotides on the formation of Tk⁺ colonies in the Ltk⁻ cells cotransfected with HSVtk Pvu II fragment

polymers(10 µg)	% inhibition
---	0
poly(dG-m ⁵ dC)	98
poly(dG-dC)	96
poly(dG-dC)-AAF	72
poly(dA-dC).(dG-dT)	54
poly(dG).poly(dC)	32
poly(dA-dT)	6

10 µg carrier DNA plus 0.2 µg PvuII fragments were used in each experiment.

to about 20% is in a Z-conformation (48,49). Data in Table II show that this polymer inhibits tk gene transfection by 72%. Interestingly, poly(dA-dC).poly(dG-dT), which converts from B to Z conformations only under very stringent conditions (50,51), showed a relatively modest inhibitory effect of 54%. On the other hand, both poly(dG).poly(dC) and poly(dA-dT).poly(dA-dT), neither of which convert into the left-handed Z-form, have any significant effect in the transfection assay. From these experiments, we conclude that there is a good correlation between the tendency of polymers to assume a Z-conformation and the ability of these polymers to inhibit Tk⁺ colony formation in Ltk⁻ cells upon transfection.

DNA uptake by Ltk⁻ cells.

A possible reason for the decreased efficiency of Tk⁺ colony formation could be due to an inhibitory effect of the Z-DNA forming polymers on the uptake of exogenous DNA by the Ltk⁻ cells. Therefore, we determined the amount of transfected DNA that is taken up by the cells in the absence and presence of the various polymers. Transfections of Ltk⁻ cells were performed using [³²P]-labeled tk DNA together with or without polymers. About 48 hours after transfection, we determined the total radioactivity in cellular DNA. Despite repeated experiments in triplicate dishes, a range of differential counts between the plates in the same group were obtained. These results are probably due to variations in transfectability of cell populations on different plates. However, it is apparent from the results presented in Table III that the uptake of exogenous DNA was not significantly affected by the various polymers used in cotransfections. We also attempted to determine the uptake of labeled probe into nuclear and cytoplasmic fractions of Ltk⁻ cells. The reproducibility of these experiments was poor, despite repeated attempts, presumably due to the variations in

Table III. Uptake of exogenous DNA by Ltk⁻ cells co-transfected with [³²P]tk gene and various polymers

polymer	cpm/plate			mean	Average %
	A	B	C		
--	4647	4884	5229	4920	100
+ 2 µg poly(dG-m ⁵ dC)	4092	4163	4415	4223	86
+ 2 µg poly(dG-dC)	4603	5562	3691	4619	94
+ 5 µg poly(dA-dT)	4302	5974	6711	5662	115
+ 5 µg poly(dG)(dC)	3331	3868	3764	3654	74

10 µg of Ltk⁻ carrier DNA and [³²P]labeled PvuII fragment of tk gene containing 6×10^5 CPM was transfected per plate in each group.

isolating the same number of nuclei in each set of experiments. Nevertheless, in all instances, no significant differences in the uptake of DNA, either in cytoplasmic or nuclear fractions, were observed in the presence or absence of the polymers (data not shown). Taken together, these results show that inhibition of Tk⁺ colony formation by potential Z-DNA polymers cannot be rationalized as a result of the differences in DNA uptake by the cells.

Transfection of Ltk⁻ cells with recombinant plasmids

Since it has been shown that cotransfected DNAs become ligated to one another once they enter the cell nuclei (52) we investigated whether potential Z-DNA sequences linked to the structural gene for tk were responsible for the inhibition of Tk⁺ colony formation. We constructed recombinant plasmids with alternating dG-dC stretches of about 20-42 bp at either the 5' or 3' ends of the tk gene. For these constructs, we created a tk⁺ derivative of the pSV2gpt vector as described in Materials and Methods (Fig. 1).

The tk⁺ derivative of pSV2gpt, designated p33tk, carries no dG-dC sequence and thus served as a control. Plasmid p31Ztk contains a stretch of about 20 bp proximal to the tk coding region and p30Ztk contains a dG-dC sequence of about 42 bp. To minimize the differences in the number of colonies formed due to the variations among the individual groups in these experiments, we have pooled and averaged the total number of colonies formed by two different concentrations of the plasmid DNAs. The results presented in Table IV show that there is no significant difference among

Table IV. Transfection of Ltk^- cells with PSV2-Gpt plasmids containing HSVtk Pvu II fragment and (dG-dC)_n inserts

Plasmid	<u>Average number of colonies and DNA per plate</u>		Total no. of colonies	Average %
	0.25 μ g	0.5 μ g		
p33tk	149	312	461	100
p312tk	128	396	524	114
p302tk	148	350	498	108
p33tk = PSV2 Gpt containing Pvu II fragment				
p312tk = p33 tk + 20 bp dG-dC insert at 5' of tk gene				
p 302tk = p33tk + 42 bp dG-dC insert at 5' of tk				

Duplicate plates were always counted to determine the average number of colonies per set of experiments using different plasmids and amount of DNAs together with 10 μ g of Ltk^- carrier DNA per plate.

these three groups in the average percentage of colony formation. Variations of 6-14% among the groups compared with the controls are typical for these types of transfection procedures. Furthermore, transfection with p272tk showed no significant difference from the assays using plasmids carrying dG-dC sequences 3' of tk (data not shown), indicating that a potential Z-DNA insert at the 3' end of the tk gene does not exert a position effect. Because an inhibition of Tk^+ colony formation by potential Z-DNA polymers was obtained upon transfecting plasmid ptk5 into Ltk^- cells (Table I, II), we next explored whether the newly constructed plasmid, p33tk, behaved similarly in analogous experiments. For these assays, the same concentration of polymers was used which yielded 50% inhibition of Tk^+ colony formation by ptk5. Table V shows that all the polymers behaved in a similar way in the cotransfection assays using both tk^+ constructs either p33tk or ptk5.

In order to characterize the Tk^+ clones that arose after transfection with the Z-DNA constructs, and prove the presence of inserted dG-dC sequences proximal to tk in these clones, we isolated colonies that were phenotypically positive for both selective markers (Tk^+ and Gpt^+). Genomic DNAs from these Tk^+ and Gpt^+ clones were digested with the restriction enzyme Bss II (G^+CGCGC), which cleaves once in the Pvu II fragment of the tk gene and also recognizes the dG-dC sequences in the plasmid. This digestion generates a fragment of about 1.4 kb when dG-dC sequences are linked at the 5' end of the tk gene. Southern blot analysis (Fig. 3) showed

Table V. Effect of various co-transfected polymers on the expression of tk⁺ gene in p33tk plasmid

p33tk (0.5 µg)	Tk ⁺ colonies			
	Plate A	Plate B	Mean	Average %
---	242	283	263	100.0
+ 2 µg poly(dG-m ⁵ dC)	119	129	124	47.0
+ 8 µg poly(dG-dC)	125	134	130	50.0
+ 8 µg poly(dG)(dC)	247	228	238	90.1
+ 8 µg poly(dA-dT)	218	237	228	87.0

Figure 3. Southern blot analysis of the genomic DNAs isolated from different clones. Hind III digested λ and Hae III digested ϕ X174 RF DNAs as markers were included in the gel; the relative positions of marker bands are indicated on the left. BssH II digested whole genomic DNA derived from a clone transfected with plasmid p31Ztk appear in lane 1. BssH II digested genomic DNAs from two different clones isolated following transfection with plasmid p30Ztk appear in lanes 2 and 3, respectively. BssH II digested genomic DNA obtained from a clone, isolated after transfection with plasmid P33tk as a control is shown in lane 4. The arrow indicates the position of the 1.4 kb fragment.

that the DNAs derived from three Tk^+ , Gpt^+ colonies transfected with either p31Ztk DNA, carrying 20 bp of dG-dC (lane 1), or with p30Ztk DNA, carrying 42 bp of dG-dC (lane 2 and 3), reveal the same 1.4 kb band. However, DNA from cells transfected with p33tk and digested with BssH II did not hybridize with the tk probe (lane 4).

From these results we can conclude that: 1) the dG-dC sequences which are physically linked to the tk⁺ gene on the constructs are maintained by the Ltk⁻ cells during transformation to a Tk^+ phenotype, and 2) the presence of linked dG-dC sequences at the 5' or 3' ends of tk does not effect the tk gene transfection frequency.

DISCUSSION

In this report, we have attempted to evaluate the effects of potential Z-DNA forming polymers on mammalian gene transfection. A precise explanation of the inhibition of tk gene transfection by potential Z-DNA conformers, when used in cotransfection studies, is hampered by a lack of detailed understanding of the mechanisms involved in eukaryotic gene transfection, stable integration and expression. The ability of Z-DNA sequences to act as either a positive or negative regulatory element has been suggested for SV-40 (22,23) and tRNA genes (25,26), respectively. The results presented in the present study indicate that inhibition of Tk^+ colony formation correlates well with the potential of different polymers to adopt the Z-DNA conformation. Poly(dG-m⁵dC).poly(dG-m⁵dC), which undergoes a B to Z transition in solution under physiological conditions (2) is considered to be one of the most potent Z-DNA conformers; this polymer showed the highest inhibitory effect. Moreover, it has been demonstrated that transfected DNAs retain their patterns of methylation after incorporation into mammalian cells (53). Hence, we assume that poly(dG-m⁵dC).poly(dG-m⁵dC) may remain methylated to a partial extent after transfection and could be in the Z-conformation within the recipient cells. Poly(dG-dC).poly(dG-dC) is somewhat less likely to assume the Z-DNA conformation and also inhibits the transfection efficiency to a lesser degree than poly(dG-m⁵dC).poly(dG-m⁵dC). By contrast, poly(dG).poly(dC) and poly(dA-dT).poly(dA-dT), which do not convert into Z-DNA had no significant effect on Tk^+ colony formation. There is conflicting evidence as to whether or not poly(dA-dC).poly(dG-dT) is able to undergo B to Z conformational changes (50,51). As demonstrated here, the poly(dA-dC).poly(dG-dT) had intermediate activity in our cotransfection assay.

Furthermore, the inhibitory effect of these polymers was found to be similar using either circular or linear forms of plasmid tk DNA molecules for transfection. We have ruled out the possibility that the polymers simply act by blocking the uptake of transfected DNAs by Ltk^- cells. Moreover, we performed toxicity tests by treating the cells with these polymers and have not seen any significant deleterious effects as determined by cell counts and morphology over a period of one week (data not shown).

In contrast to the results obtained in the cotransfection studies, we found that 20-42 base pair long stretches of alternating dG-dC sequences inserted into recombinant plasmids at the 5' or 3' end of the Pvu II tk fragment had no effect on the transfection frequency obtained with the tk gene. It is probable that multiple copies of the construct were integrated into the chromosomes of the recipient cells which, in turn, became Tk^+ and were selected for further study. Our Southern blot analyses showed that the dG-dC stretches remained physically linked to the integrated tk gene in these clones, because we observed a 1.4 kb band using [^{32}P]-labeled Pvu II tk fragment as the probe. One might argue that some plasmids may have been cleaved in vivo during the transfection process so as to separate the tk gene and the Z element. The result of such cleavage could be that the tk gene(s) conferring the positive phenotype are not the same one(s) detected by BssH II cleavage. However, the Southern blot of genomic DNAs obtained from isolated Tk^+ clones and cleaved with BssH II showed a single 1.4 kb band, the anticipated size of the plasmid segment from dG-dC sequences to the cleavage site within the tk gene (Figure 3). If copies of the disrupted tk constructs were integrated into the cellular genome, which no longer carry dG-dC sequences proximal to tk, then we would have detected other fragments of various sizes. Hence, in these isolated clones, the tk gene linked to the dG-dC segments were expressed and conferred the positive phenotype.

An important difference between these two types of transfection experiments should be emphasized. In the cotransfection experiments, high molecular weight polynucleotides were used, whereas the tracts of alternating dG-dC sequences were relatively short in our recombinant plasmids. It has been previously reported (3) that longer segments of dG-dC (>50 bp) cannot be cloned and maintained in plasmids. The most plausible explanation for the inhibition of colony formation by potential Z-DNA polymers is that the cotransfected polymers prevent stable integration of the

tk gene into the cellular genome of Ltk⁻ cells. Presumably, when the dG-dC sequence is already inserted in the transfected plasmids, as in the case of our constructs, they do not interfere with stable integration or transcription of the gene. Perhaps, the short stretches of dG-dC sequences in our constructs remain in the B form under the experimental conditions, and thus do not exert a significant inhibitory effect (29). Our results do not exclude the possibility that the cotransfected polymers are stably integrated into the cellular genome together with the tk gene and that because of their Z-conformation, they interfere with transcription of the gene. However, we think that this is unlikely. Thus, further studies concerning the possible effects of variously sized stretches of Z-DNA on the process of gene expression are required, and these are in progress.

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