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Biochemistry

Enhanced expression of the bacterial chloramphenicol acetyltransferase gene in mouse cells cotransfected with synthetic polynucleotides able to form Z-DNA

(calcium phosphate coprecipitation/transient expression/positive regulatory elements/eukaryotic transcription)

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ABSTRACT **Recent studies have demonstrated that the left-handed, Z-DNA conformation is favored in polymers containing alternating purine/pyrimidine sequences that can exist *in vivo* and may play a role in gene expression. On the basis of this assumption, we have studied the effect of various cotransfected polynucleotides on the transient expression of the chloramphenicol acetyltransferase (CAT) gene in thymidine kinase-deficient murine L cells. Cotransfections were performed by calcium phosphate coprecipitation of CAT gene plasmids with various polymers, and the CAT enzymatic activity was measured in cell lysates after 48 hr. About 2- to 10-fold stimulation of CAT gene expression was observed when the cells were cotransfected with 10 μg (per 10-cm culture dish) of plasmid pSV2cat, which contains simian virus 40 (SV40) promoter and enhancer sequences, and 2–10 μg of polymers that can form Z-DNA, such as poly(dG-m^{5}dC)·poly(dG-m^{5}dC) or poly(dG-dC)·poly(dG-dC), as compared to transfection with pSV2cat alone. Further, enhanced CAT gene expression was also observed when cotransfections were performed with these polymers and two other plasmid vectors, one containing the SV40 promoter but no enhancer and the other lacking any SV40 regulatory sequences. However, poly(dA-dC)·poly(dG-dT), which can form Z-DNA, did not induce any stimulation. Similarly, no or very little stimulation was observed after cotransfection of pSV2cat with either poly(dG)·poly(dC) or poly(dA-dT)·poly(dA-dT), which do not adopt the Z conformation. These results suggest that certain polynucleotides may enhance transcription of the CAT gene.**

The discovery that some alternating purine and pyrimidine DNA sequences may assume a left-handed Z-DNA conformation under certain conditions (1–3) raised the probability that the transition from the usual right-handed, B form to a left-handed, Z structure may have an important biological function (4, 5). By use of Z-DNA-specific antibodies, potential Z-DNA-forming regions have been detected in the chromosomes of various species (6–9). The detection of such Z-DNA sequences in 5′ or 3′ noncoding regions and within introns of a number of cloned eukaryotic genes has suggested that the Z conformation may play a role in the regulation of gene expression (10–12). In the simian virus 40 (SV40) enhancer region, 8-base-pair (bp) stretches of alternating purines and pyrimidines that may have the potential to form Z-DNA have been assumed to be involved in this process (13, 14). DNA in the Z conformation is a relatively less active template than the one in the right-handed, B form when transcribed by *Escherichia coli* RNA polymerase (15). Similarly, when an inserted d(C-G)$_{16}$ sequence was inverted to the left-handed, Z form in a plasmid, it did not serve as a template for transcription (16). However, the blockage of transcription was not observed when this alternating d(C-G) sequence was replaced by a d(T-G)$_{21}$·d(C-A)$_{21}$ insert. On the other hand, poly(dT-dG)·poly(dC-dA), which has the potential to adopt the Z conformation when inserted into a chloramphenicol acetyltransferase (CAT) gene expression plasmid vector, enhanced the transient expression of this gene (17). Further, the *Ustilago* rec-1 protein, which is involved in heteroduplex formation in recombination, appears to have a strong Z-DNA binding site, and it has been proposed that DNA in the Z conformation may play a key role in promoting this process (18).

We have recently reported (19) that certain potential Z-DNA-forming polymers, such as poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC), when cotransfected with the herpes simplex virus thymidine kinase (TK) gene, can inhibit the level of TK gene transfection by reducing the number of TK$^+$ transformed colonies. We presumed that these cotransfected polymers may have blocked the stable integration of this gene into the genomic DNA of TK-deficient L (LTK$^-$) cells. Similarly, it has been reported (20) that interferon also prevents stable integration and expression of the transfected TK gene, whereas it does not prevent transient expression of this gene in unintegrated form. Therefore, to avoid the necessity of stable integration of the transfected gene, and to be able to analyze the direct effect of Z-DNA-forming polymers on transcription, we have assayed the transient expression of the CAT gene in LTK$^-$ cells in this study.

In particular, we have examined the effect of various polynucleotides that may or may not assume Z conformation on CAT gene expression, when cotransfected with a CAT gene vector either containing SV40 promoter and enhancer (pSV2cat) or lacking any SV40 regulatory sequences (pSV0cat). We have also used CAT plasmids containing only SV40 promoter regions (pCAT3). The results indicate that poly(dG-dC)·poly(dG-dC) or poly(dG-m^{5}dC)·poly(dG-m^{5}dC), both of which are potential Z conformers, stimulate CAT gene expression. In contrast, poly(dA-dC)·poly(dG-dT), which may also assume the Z conformation, did not show any stimulation. However, poly(dG)·poly(dC) and poly(dA-dT)·poly(dA-dT) can neither form Z-DNA structure nor stimulate CAT gene expression.

MATERIALS AND METHODS

Cells and Transfection Procedure. Mouse LTK$^-$ cells (obtained from R. Axel, Columbia University, New York)

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Abbreviations: TK, thymidine kinase; LTK$^-$, TK-deficient murine L cells; CAT, chloramphenicol acetyltransferase; SV40, simian virus 40; AAF, acetylaminofluorene; bp, base pair(s).

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were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) calf serum and kept at 37°C with 5% CO_2. The cotransfection procedure was performed by the calcium phosphate precipitation method (21) as described (19). For comparison, we also used a modified DEAE-dextran transfection procedure (22) in some experiments. About 10 μg of plasmid DNA and various amounts of synthetic double-stranded polymers (Pharmacia P-L Biochemicals) were used for cotransfections on each 10-cm dish. After 48 hr of transfections, the cells were harvested for CAT assay.

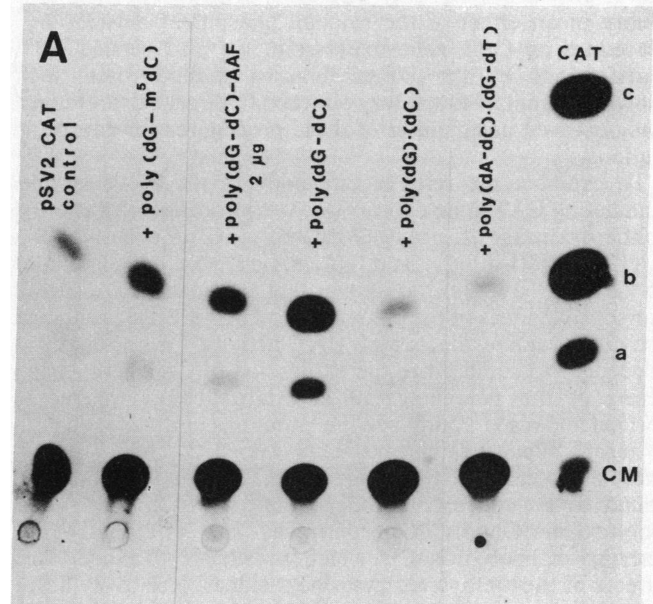
Recombinant Plasmids and DNA Isolation. The recombinant plasmids pSV2cat and pSV0cat were generously provided by B. Howard. In pSV2cat plasmids, the SV40 promoter and 72-bp enhancer regions are present (23); in pSV0cat plasmids, all of the SV40 early promoter regions, including enhancer sequences, have been deleted (24). Plasmid pCAT3 (a gift from J. Shoul, Weizmann Institute of Science, Israel) is a construct analogous to pA10-CAT2, in which most of the SV40 enhancer regions have been deleted but the promoter elements have been retained (25). The plasmid DNAs were purified by chromatography on Bio-Gel A-15m (Bio-Rad) and checked by electrophoresis in 1% agarose gel before transfections.

Synthetic Polydeoxyribonucleotides. These double-stranded polymers were obtained from Pharmacia P-L Biochemicals. Sedimentation coefficients ($s_{20,w}$) ranged mainly from 7 to 10. Poly(dG-dC)·poly(dG-dC)-AAF, in which ≈28% of the guanosine residues were modified at C-8 by reaction with *N*-acetoxy-2-acetylaminofluorene (28), was prepared in our laboratory.

CAT Assay. The procedure for CAT assay was modified from Gorman *et al.* (23). About 48 hr posttransfection, cells from a 10-cm dish were washed three times with phosphate-buffered saline. To each plate, 1 ml of Tris collection buffer (0.04 M Tris·HCl, pH 7.4/1 mM EDTA/0.15 M NaCl) was added and left for 5 min at room temperature. The cells were then collected in a microcentrifuge tube by scraping and pelleted by centrifugation. The pellet was then dispersed by Vortex mixing in 100 μl of 0.25 M Tris·HCl (pH 7.8), and a cell lysate was prepared by three freeze–thaw cycles. Enzyme assay mixtures contained (in a final volume of 150 μl) 50 μl of cell lysate, 70 μl of 1.0 M Tris·HCl (pH 7.8), 9 μl of H_2O, 1 μl of ethanol containing [^{14}C]chloramphenicol (about 0.2 μCi, New England Nuclear; 1 Ci = 37 GBq), and 20 μl of 4 mM acetyl-coenzyme A (Pharmacia P-L Biochemicals). Incubation for 1.5 hr at 37°C provided adequate CAT enzymatic activity in the lysates of the transfected cells. Chloramphenicol and its derivatives in this reaction mixture were then extracted with 1 ml of buffer-saturated ethyl acetate, and the organic phase was dried down in a Speed Vac concentrator (Savant Instrument). The samples were resuspended in 25 μl of ethyl acetate and spotted on a silica gel TLC plate (Baker Flex, cat. no. 2738-E10). After development in chloroform/methanol (95:5, vol/vol) for 1.5 hr, the plate was exposed to Kodak XAR-2 film. For quantitative measurement, the spots corresponding to appropriate acetylated forms were cut out from the TLC plate and their radioactivity was measured by liquid scintillation spectroscopy.

RESULTS

Effect of Various Cotransfected Polymers on CAT Gene Expression with pSV2cat Vectors. To determine the effect of different polynucleotides on transient CAT gene expression, we cotransfected a variety of polymers that can or cannot assume Z-DNA conformation. Cotransfections of pSV2cat with three polymers that can assume Z conformation, poly(dG-m^{5}dC)·poly(dG-m^{5}dC) (8 μg), poly(dG-dC)·poly(dG-dC) (8 μg), or poly(dG-dC)·poly(dG-dC)-AAF (2 μg), resulted in CAT gene expression that was enhanced 3-, 13-, or 4-fold, respectively, relative to that obtained by transfection of pSV2cat alone (Fig. 1). On the other hand, poly(dG)·poly(dC), which cannot assume the Z conformation, showed no remarkable stimulatory effect as compared to the control. Similarly, no stimulation was observed when cotransfections were performed with poly(dA-dC)·poly(dG-dT), which can



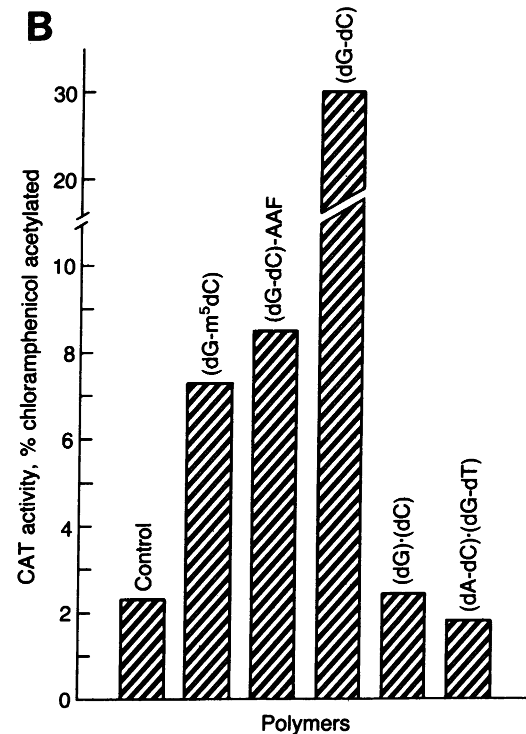


FIG. 1. Expression of CAT activity in LTK$^-$ cells cotransfected with pSV2cat (10 μg) and various polymers (8 μg) as indicated, except for poly(dG-dC)·poly(dG-dC)-AAF (2 μg). For brevity, single-stranded designations are used to represent some of the double-stranded polynucleotides. (*A*) Autoradiogram of thin-layer chromatography (TLC) showing chloramphenicol (CM) and its acetylated forms, chloramphenicol 1-acetate (a), chloramphenicol 3-acetate (b), and chloramphenicol 1,3-diacetate (c). A commercial CAT enzyme (≈2.5 units, Sigma) was used as a positive control (lane at right). (*B*) Histogram showing the relative CAT activity calculated from radioactivity in the acetylated forms (a plus b) of chloramphenicol.

undergo B-to-Z transition under certain conditions (26, 27). Comparing the data in Figs. 1*B* and 2, it appears that 2 μg of poly(dG-dC)·poly(dG-dC)-AAF, which is the strongest Z conformer (28, 29) among these polymers, is capable of producing almost 4-fold stimulation of CAT gene expression, as compared to only ≈2-fold stimulations obtained by the same concentration of cotransfected poly(dG-dC)·poly(dG-dC) or poly(dG-m^{5}dC)·poly(dG-m^{5}dC). To rule out the possibility of an effect of the calcium phosphate transfection procedure on CAT gene expression, we also carried out transfections by the DEAE-dextran method (data not shown). A similar stimulatory effect on CAT gene expression was observed using either of these procedures for cotransfections.

To evaluate the relative efficiency of the polymers in stimulating CAT gene expression, we cotransfected various concentrations of poly(dG-dC)·poly(dG-dC), poly(dG-m^{5}dC)·poly(dG-m^{5}dC), and poly(dA-dT)·poly(dA-dT) with pSV2cat. [Shaw (30) has shown that for quantitative comparisons, analysis of the chloramphenicol 3-acetate products provides valid estimates of CAT activity. We used this procedure to standardize the expression of CAT activity in terms of the percentage of acetylated chloramphenicol formed and also to minimize the variations in transfections.] When pSV2cat was cotransfected with 2, 5, and 10 μg of these polymers, the stimulation of CAT gene expression was found to be concentration-dependent (Fig. 2). However, poly(dG-m^{5}dC)·poly(dG-m^{5}dC) was less effective than poly(dG-dC)·poly(dG-dC), which presumably reflects the effects of the methylated cytosine residues. Relatively little stimulation was seen with poly(dA-dT)·poly(dA-dT) even when 10 μg of the polymer was used for cotransfection.

Cotransfection of Polymers and pCAT3. We assumed that the enhancement of CAT gene expression by cotransfected polymers might be related to an effect of these polymers on the SV40 regulatory sequences in the pSV2cat plasmid. Therefore, to evaluate the possible role of the polymers and the SV40 enhancer and/or promoter sequences, we used two other plasmid constructs for cotransfections. One of these, pCAT3, has SV40 promoter sequences but no enhancer sequences; the other, pSV0cat, lacks both enhancer and promoter SV40 sequences. When 10 μg of pCAT3 DNA and 8 μg of polymer were cotransfected, poly(dG-m^{5}dC)·poly(dG-m^{5}dC) and poly(dG-dC)·poly(dG-dC) had stronger stimulatory effects than did poly(dG)·poly(dC) (Fig. 3). The stimulatory effect of poly(dG-dC)·poly(dG-dC) was greater than that of poly(dG-m^{5}dC)·poly(dG-m^{5}dC), as we observed in the cotransfection experiments with pSV2cat plasmids. As expected, the CAT activity in LTK$^-$ cells transfected with pCAT3 DNA alone (Fig. 3) was about one-fourth that obtained with transfection of pSV2cat alone (Fig. 1*B*). This is due to the lack of SV40 enhancer regions in pCAT3, which play a key rôle in CAT gene regulation in these plasmids (23–25). However, the relative stimulation of CAT gene expression when pCAT3 was cotransfected with the poly-

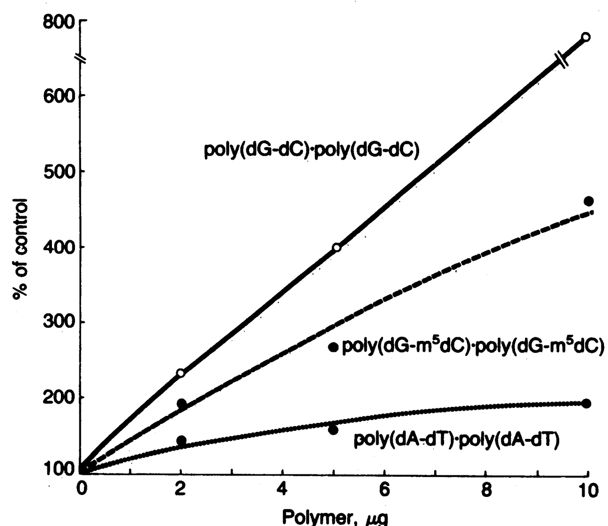
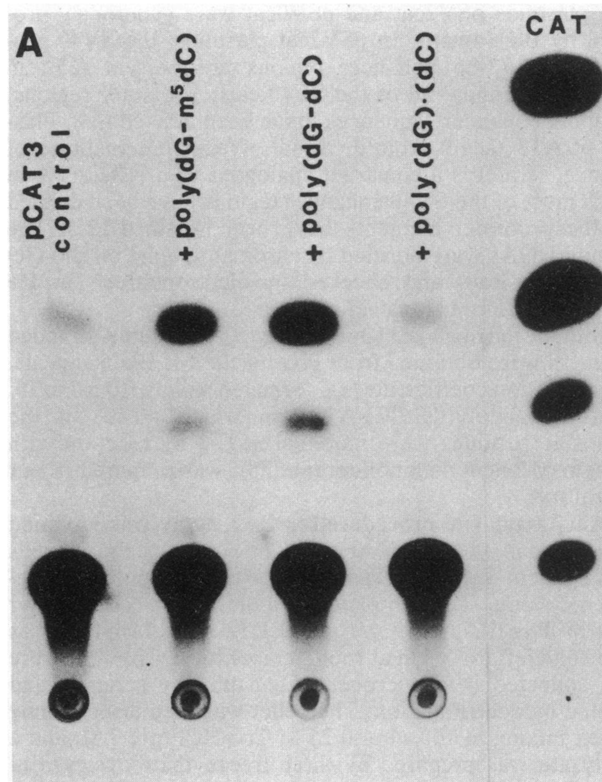


FIG. 2. Effect of various concentrations of poly(dG-dC)·poly(dG-dC) (○—○), poly(dG-m^{5}dC)·poly(dG-m^{5}dC) (●---●), and poly(dA-dT)·poly(dA-dT) (●·····●) on transient CAT gene expression in LTK$^-$ cells assayed 48 hr after transfections. Data represent an average of two experiments.



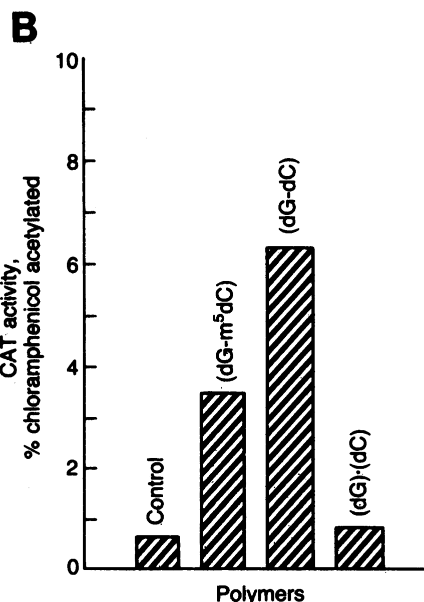
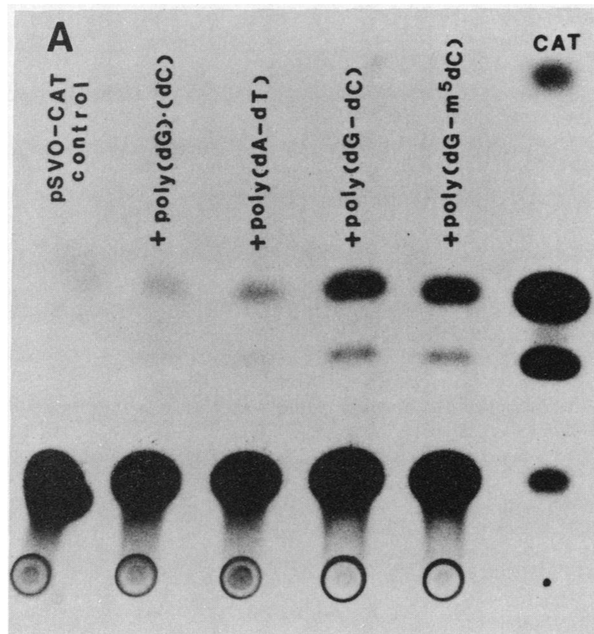


FIG. 3. CAT gene expression in LTK$^-$ cells cotransfected with enhancer-minus plasmid pCAT3 (10 μg) and various polymers (8 μg). (*A*) Autoradiogram of TLC. (*B*) Histogram showing the relative CAT activity.

mers was approximately the same as that obtained by cotransfection of pSV2cat with the polymers.

Cotransfection of Polymers and pSV0cat. Since we observed that certain polymers can induce a stimulatory effect on CAT gene expression in enhancer-minus pCAT3 plasmid, we were interested to learn about the effect of these polymers on pSV0cat as well. This plasmid is lacking both eukaryotic promoter and eukaryotic enhancer sequences. Because the expression of the CAT gene on this plasmid is almost nonexistent, we used a high concentration (20 μg) of various polymers in cotransfection experiments to evaluate their effects. Although transfection with pSV0cat alone resulted in little CAT activity, cotransfections with some of the polymers produced significant CAT expression (Fig. 4*A*). About 7-fold stimulation of CAT gene expression was observed after cotransfection of pSV0cat with poly(dG-dC)·poly(dG-dC) or poly(dG-m^{5}dC)·poly(dG-m^{5}dC), whereas poly(dG)·poly(dC) or poly(dA-dT)·poly(dA-dT) did not have any significant effect as compared to the control (Fig. 4*B*). The data also show that in these experiments poly(dG-m^{5}dC)·poly(dG-m^{5}dC) was as potent as poly(dG-dC)·poly(dG-dC), which was not observed before. Perhaps this was due to the very high polymer concentration, at which the stimulation by poly(dG-dC)·poly(dG-dC) may have reached the plateau level.

To evaluate the nature of stimulatory effects on this promoterless, enhancerless plasmid, we cotransfected various concentrations of poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) with pSV0cat DNA. The results (Fig. 5) show that the stimulation of CAT gene expression by cotransfected poly(dG-dC)·poly(dG-dC) is concentration-dependent. A relatively similar concentration-dependence was observed when cotransfections were performed with various concentrations of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) (data not shown). Moreover, no significant CAT activity was observed in LTK$^-$ cells transfected with control pSV0cat DNA as compared to the mock-transfected cells (Fig. 5).



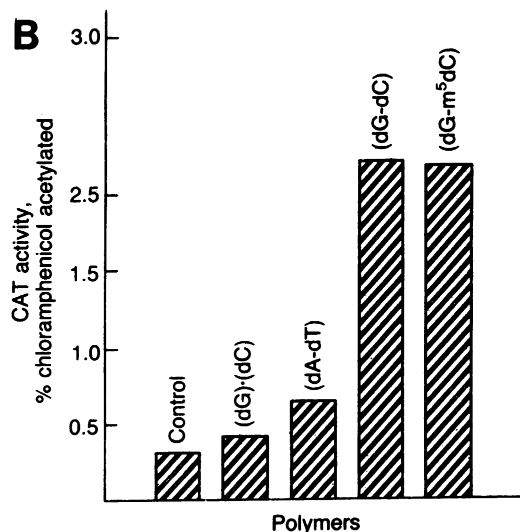


FIG. 4. CAT gene expression in LTK$^-$ cells cotransfected with pSV0cat (10 μg) and different polymers (20 μg). (*A*) Autoradiogram of TLC. (*B*) Histogram indicating the relative CAT activity.

DISCUSSION

An understanding of the mechanism of enhancement of gene expression by specific polynucleotides is limited by lack of knowledge of the nature of transcriptional activation in eukaryotic cells in general. Significant advancement has been made in the identification of specific DNA sequences involved in the initiation of transcription by RNA polymerase II *in vivo* (31). Several elements have proven to be important for the activation of eukaryotic transcription. In SV40, at least one of the 72-bp repeats is thought to act in *cis* to activate the early genes (32–34).

We investigated the transient expression of bacterial CAT gene, which can be transcribed in mammalian cells when under the control of eukaryotic promoter and enhancer sequences, such as those of SV40 (23, 24). Our results show (Fig. 1) that certain double-stranded polydeoxyribonucleotides stimulate CAT gene expression in LTK$^-$ cells when cotransfected with pSV2cat, which contains SV40 promoter and enhancer sequences. A similar stimulation was observed when these polymers were cotransfected with the enhancer-minus plasmid pCAT3 or the plasmid pSV0cat, which has neither enhancer nor promoter sequences (Figs. 2 and 3). Although the control level of CAT activity is much lower with pCAT3 and pSV0cat than with pSV2cat, the relative enhancement of CAT gene expression induced by the polymers is similar for the three plasmids. Our results indicate that the polymers that are able to form Z-DNA, except for poly(dG-

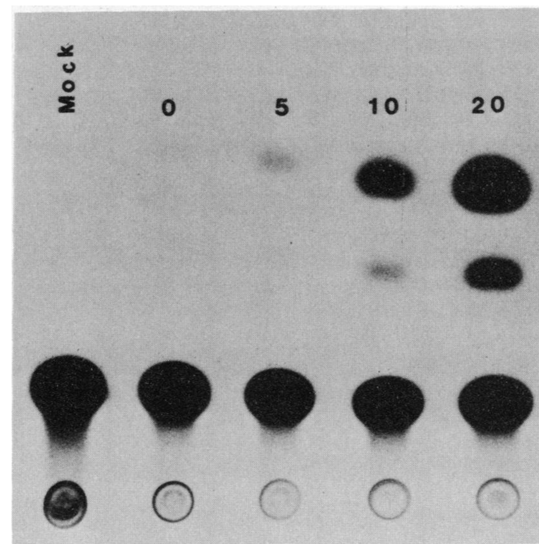


FIG. 5. Effect of various concentrations of poly(dG-dC)·poly(dG-dC) cotransfected with pSV0cat DNA (10 μg) on CAT gene expression in LTK$^-$ cells. The amount (μg) of polymer is indicated at the top of each lane. Lane at left shows CAT assay of mock-transfected (no DNA) cells.

dT)·poly(dA-dC), stimulate CAT gene expression. In contrast, poly(dG)·poly(dC) and poly(dA-dT)·poly(dA-dT), which cannot form Z-DNA, failed to exert any noticeable stimulatory effect. Since none of these polymers significantly affect the uptake of exogenous DNA into LTK$^-$ cells during transfections (19), we can exclude the possibility that the stimulatory polymers simply facilitate a higher level of DNA transfection. Moreover, if the stimulatory polymers acted only by facilitating the transfectional mechanism(s) or enhancing the DNA uptake, then it is most unlikely that much stimulation would be observed with pSV0cat, which is not transcribed in mammalian cells (Fig. 4*A*).

Our results suggest that the properties of the stimulatory polymers are similar to those of viral enhancers and/or promoters. The mechanism of enhancement by these polymers is not known. One possibility is that some of these cotransfected polymers, upon entering the LTK$^-$ cells, become linked to the CAT vectors and, by undergoing recombination (35, 36), affect the gene expression. However, if this were the case, the stimulatory effect could be similar to that observed by Hamada *et al.* (17) when they linked poly(dT-dG)·poly(dC-dA) to a CAT gene plasmid vector. In contrast to their results, we have not seen any stimulation of CAT gene expression after cotransfection of linear poly(dT-dG)·poly(dC-dA). It is possible that the short stretches of poly(dT-dG)·poly(dC-dA) inserted into a supercoiled plasmid used in the experiments of Hamada *et al.* might have a different conformation than the cotransfected polymers used in our experiments. Another possibility is that some repressor-like cellular factors or Z-DNA binding proteins (37, 38) may specifically recognize segments in some of these cotransfected synthetic DNAs which, in turn, promote the transcriptional efficiency of the CAT gene. This could be another reason why certain polymers are capable of stimulating CAT gene expression in LTK$^-$ cells. In spite of all the probable implications concerning Z-DNA conformation in the modulation of CAT gene expression, we cannot exclude the possibility that some other unknown factor(s) may be involved in this process.

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