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Enhanced Gene Expression by the Poly(dT-dG) · Poly(dC-dA) Sequence

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The sequence poly(dT-dG) · poly(dC-dA) (TG-element) is a ubiquitous component of eucaryotic genomes and has the potential to adopt a left-handed DNA conformation (Z-DNA). In this report, we have tested the hypothesis that the TG-element can modulate gene expression. Human genomic DNA fragments (1 to 1.5 kilobases) containing a $(dT\text{-}dG)_n \cdot (dC\text{-}dA)_n$ tract (30, 40, or 50 base pairs) or chemically synthesized $(dT\text{-}dG)_n \cdot (dC\text{-}dA)_n$ fragments (50 to 130 base pairs) were inserted in the pSV2-cat (simian virus 40 enhancer plus) or pA_{10}-cat (enhancer minus) expression vector plasmid. These constructs were transfected into CV-1 cells or HeLa cells, and their transcription was monitored by assaying chloramphenicol acetyltransferase activity. The results showed that pSV2-cat with the TG-element and pA_{10}-cat with the TG-element synthesized more chloramphenicol acetyltransferase activity (2 to 10 times, depending on the location of the TG-element) than did parental pSV2-cat and pA_{10}-cat DNAs, respectively. Furthermore, the TG-element appeared to have characteristics similar to those of viral enhancers: (i) the TG-element enhanced transcription from a distance, (ii) its closer location to the promoter was more effective, and (iii) its orientation was not crucial. However, its enhancer-like activity was much weaker than that of the simian virus 40 enhancer, and, unlike many viral enhancers, it was equally active in monkey and in human cells. These results suggest that the TG-element may influence the expression of cellular genes.

The alternating copolymer, poly(dT-dG) · poly(dC-dA), is a highly evolutionarily conserved DNA component of eucaryotic genomes (8, 9). The human genome, for example, contains ca. 10^5 copies of 20- to 60-base-pair (bp) $(dT\text{-}dG)_n \cdot (dC\text{-}dA)_n$ tract randomly distributed (10). This DNA component (TG-element) has received considerable attention because it can adopt the left-handed DNA conformation (Z-DNA) (11, 18). In vitro studies have shown that the poly(dT-dG) · poly(dC-dA) sequence can form two different DNA conformations (B- and Z-forms), depending on environmental factors such as the salt concentration and the superhelical density of plasmids in which they are located (11, 18). Furthermore, when in the Z-form, the $(T\text{-}G)_n \cdot (C\text{-}A)_n$ tract is susceptible to the single-stranded endonuclease (S1) (10, 25).

Recent progress in studying transcriptional regulation has identified several classes of DNA sequences that seem to be important for the regulation of transcriptional processes (24). Although the mechanism by which these DNA elements regulate transcription is not known, the simian virus 40 (SV40) enhancer (12) and the 5′-upstream sequences of certain eucaryotic genes (15, 17) are known to have unusual structures in vivo as detected by hypersensitivity to DNase I or S1 nuclease. Such structures, observed in chromatin, are often preserved in deproteinized DNA when the sequence is under torsional stress (26). Z-DNA segments in the SV40 enhancer sequences have been demonstrated (19), and there is currently strong interest in the possibility that the presence of Z-DNA affects gene expression.

In the following experiments, we test the hypothesis that the TG-element may modulate gene transcription. Our approach has been to construct recombinant plasmids in which the TG-element is linked to the SV40 promotor and the coding sequence for the bacterial enzyme, chloramphenicol acetyltransferase (CAT), and to determine the level of CAT activity in extracts of cells after transfection with these constructs. We find that the TG-element increased CAT gene expression as much as 10 times over controls. These results raise the possibility that TG-elements dispersed in the genome may influence the expression of cellular genes.

MATERIALS AND METHODS

Construction of recombinant plasmids. (i) pSV2-cat derivatives. Three human genomic fragments containing a TG-element (fragments 1, 3, and 4) were inserted at the *Bam*HI site of pSV2-cat. Fragment 1 is a *Bam*HI 1.4-kilobase (kb) human DNA with 30 bp of $(dT\text{-}dG)_n \cdot (dC\text{-}dA)_n$ derived from λHa-314 (9). Fragment 3 is a *Sau*3A 1.0-kb DNA consisting of 0.35 kb (residue 4045 to 30) from pBR322 and 0.7 kb of human DNA; 50 bp of $(dT\text{-}dG)_n \cdot (dC\text{-}dA)_n$ is included in the 0.7-kb human DNA region. Fragment 4 is a *Sau*3A 1.4-kb human DNA containing 40 bp of imperfect $(dT\text{-}dG)_n \cdot (dC\text{-}dA)_n$ (4 bases out of alternation). Fragment 4 and 0.7 kb of human DNA in fragment 3 are originated from λTG-3 and λTG-1, respectively, which are recombinant phages randomly isolated from the human DNA library by the hybridization to poly(T-G) · poly(C-A) (10). Each fragment except fragment 4 was cloned in both orientations (see Fig. 1 for details).

For construction of $pSV2\text{-}cat_{1,2}$ 1.0- to 1.5-kb fragments were isolated from the *Bam*HI digest of total HeLa DNA and ligated to *Bam*HI-digested pSV2-cat. Two clones that did not hybridize to poly(dT-dG) · poly(dC-dA) were randomly chosen and used for transfection.

(ii) pA_{10}-cat derivatives. Construction of pA_{10}-cat has been described previously (13). Fragments 1, 3, and 4 were inserted in both orientations, either at a *Bgl*II site or at a *Bam*HI site of pA_{10}-cat. p5′-4a designates a recombinant in which fragment 4 is inserted at a 5′ site (the *Bgl*II site) in an orientation such that $(T\text{-}G)_n$-strand is the sense strand of the CAT coding sequence. p5′-4b has fragment 4 at the same

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place but in the opposite orientation. p3'-4a and p3'-4b have fragment 4 at the 3' site (the *Bam*HI site) in two different orientations. Recombinant plasmids containing fragment 1 or 3 are designated in the same way.

For construction of pA_{10}-cat derivatives containing only a $(dT\text{-}dG)_n \cdot (dC\text{-}dA)_n$ insert, poly(dT-dG) · poly(dC-dA) (Boehringer Mannheim Biochemicals) was first digested with S1 nuclease. The S1 digest was separated on a 10% acrylamide gel, and the 40- to 150-bp region was recovered. This DNA was ligated to *Bam*HI linkers and then ligated to *Bam*HI-digested pA_{10}-cat DNA. Clones containing a $(dT\text{-}dG)_n \cdot (dC\text{-}dA)_n$ insert were selected by hybridization to poly(dT-dG) · poly(dC-dA). The size of the inserted $(dT\text{-}dG)_n \cdot (dC\text{-}dA)_n$ was determined by *Bam*HI digestion, followed by polyacrylamide gel electrophoresis. The recombinant plasmids thus obtained were designated as p3'-50, p3'-60, and p3'-70, etc., according to the size of the insert.

The inserts in p3'-50, p3'-60, p3'-70, p3'-80, p3'-120, and p3'-130 were excised by digestion with *Bam*HI and isolated. Each DNA was separately ligated to the *Bgl*II-digested pA_{10}-cat DNA. Clones were first selected by hybridization to poly(T-G) · poly(C-A). The size of the inserted DNA was confirmed on an agarose gel. Recombinant plasmids were designated p5'-50, p5'-60, p5'-70, p5'-100, and p5'-130, according to the size of the $(T\text{-}G)_n \cdot (C\text{-}A)_n$ insert. The nucleotide sequence of the inserts in p5'-50a, p5'-50b, p5'-60a, and p5'-60b was determined from an *Nco*I site located ca. 110 bp downstream from the *Bgl*II site, which indicated that at least 40 bp from the 3' end of the insert was precisely alternating $(dT\text{-}dG)_n \cdot (dC\text{-}dA)_n$.

Although sequences of the inserts in other plasmids have not been determined, it is most likely that they are uniformly $(dT\text{-}dG)_n \cdot (dC\text{-}dA)_n$, because the stability of hybridization to poly(dT-dG) · poly(dC-dA) was dependent on the size of the insert and because poly(dT-dG) · poly(dC-dA) was initially treated with S1 nuclease to remove base mismatching.

DNA preparation for transfection. All recombinant plasmid DNAs were propagated in *Escherichia coli* (C-600). The DNA was prepared from cleared lysates and first purified by gel filtration. Form I DNA was isolated after centrifugation in the presence of CsCl and ethidium bromide, dialyzed extensively against 15 mM NaCl–1.5 mM sodium citrate, and used for transfection.

Transfection and CAT assay. Detailed procedures for DNA transfection and the CAT assay have been described previously (6). CV-1 or HeLa cells were plated 1 day before transfection. Cells were diluted such that cells from one confluent 10-cm petri dish were split 1:10. Duplicate dishes were prepared for each DNA. A 10-μg amount of either control DNA (pSV2-cat or pA_{10}-cat) or an equivalent of test DNA (pSV2-cat derivatives or pA_{10}-cat derivatives) were transfected to one dish by the $CaPO_4$ procedure. At 4 h after transfection, cells were treated with 12.5% (vol/vol) glycerol for 2 min for CV-1 or 30 s for HeLa cells and further incubated with culture medium for 40 to 44 h. For the assay of CAT activity, cells were harvested from the duplicate plates and suspended in 200 μl of 0.25 M Tris · hydrochloride (pH 7.8). Cell extracts were prepared by repeated (five times) freezing and thawing of cells. Samples of cell extracts were incubated with 0.3 μCi of [^{14}C]chloramphenicol (New England Nuclear Corp.). Acetylated and unacetylated forms of chloramphenicol were separated by thin-layer chromatography. To quantitate the CAT activity, the regions corresponding to acetylated and unacetylated forms were cut out from the thin-layer plates, and the radioactivity was determined. CAT activity is expressed as percent acetylated chloramphenicol. Since cell number plated in a dish varies in each experiment, CAT activity in two different experiments cannot be compared. However, CAT activity of a given DNA relative to that of a control (pA_{10}-cat or pSV2-cat) was constant.

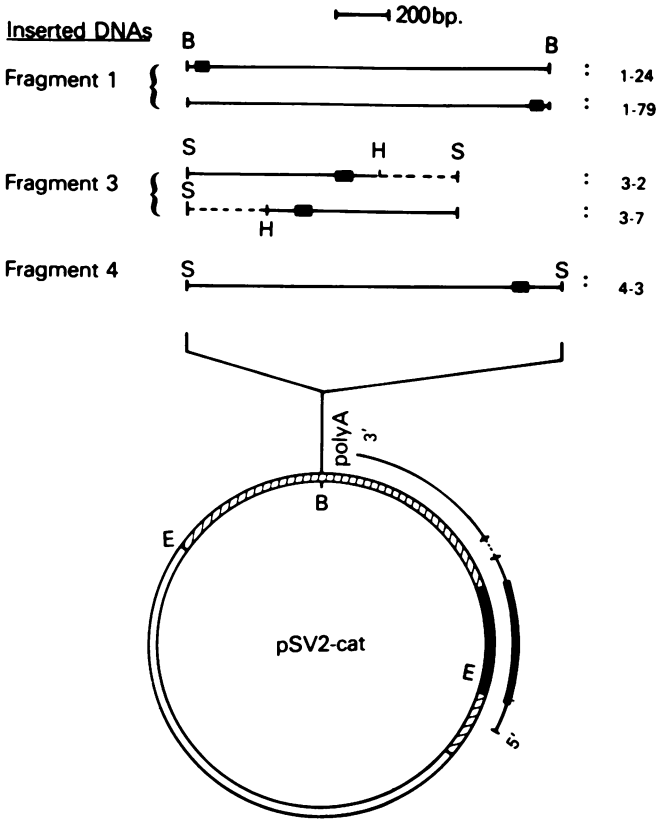

FIG. 1. Schematic diagram of pSV2-cat and its derivatives. The functional map of pSV2-cat is shown as a circle. Symbols: ▭, pBR322; ▨, SV40; ▬, CAT coding sequence (6). mRNA transcribed from pSV2-cat is indicated outside the circle. The three human genomic fragments containing a Z_{TG}-element were inserted (in two orientations except fragment 4) at a *Bam*HI site of pSV2-cat, and the designations of the resulting plasmids are shown above. Symbols: ├──┤, human DNA; ├ ─ ─ ┤, pBR322; ▬, the TG-element.

RESULTS

Enhanced CAT gene expression by human genomic fragments with a TG-element. (i) In pSV2-cat. pSV2-cat (6) is a recombinant plasmid consisting of an ampicillin-resistant gene and a replication origin from pBR322, a replication origin and polyadenylic acid addition signal sequence from SV40, and a CAT gene from Tn*9* (Fig. 1). When pSV2-cat DNA is transfected to cultured cells, the DNA is transcribed in vivo from the SV40 early promotor through the CAT gene, producing mRNA with polyadenylic acid that is translated into CAT protein (Fig. 1). This system provides a number of advantages in assaying gene activity. The production of CAT in eucaryotic cells can be measured sensitively and accurately. Because the CAT activity is apparently absent from eucaryotic cells, the background level of gene expression is effectively zero. Furthermore, in previous studies (5) the level of CAT activity in cell extracts has been found to correlate with the mRNA level. The strategy we used here was to construct various pSV2-cat plasmids with the TG-element, to transfect these DNAs to monkey cells,

and to determine their transcriptional efficiency in comparison with that of the parental DNA by assaying CAT activity.

First, we prepared five plasmids with TG-elements by inserting three human DNA fragments, each containing a TG-element, in both orientations at a *Bam*HI site of pSV2-cat (Fig. 1) (the alternative orientation of pSV2-cat$_{4\text{-}3}$ was not constructed). Since the *Bam*HI site is 0.2 kb downstream from the polyadenylic acid addition site and 3.5 kb upstream from the SV40 promotor, insertion of foreign DNA fragments at this site should not disturb the transcriptional unit. The three DNA fragments inserted were randomly isolated from the human DNA library by hybridization to poly(T-G) · poly(C-A); their nucleotide sequences have been partially determined (10). Fragment 1 is 1.4 kb with a (dT-dG)$_{15}$ tract. Fragment 2 is 0.7 kb with a (dT-dG)$_{25}$ tract and 0.3-kb pBR322 sequence. Fragment 3 is 1.4 kb containing an imperfect (dT-dG)$_{20}$ tract. These three DNA fragments do not have any significant sequence homology other than a (dT-dG)$_n$. We also constructed pSV2-cat$_1$ and pSV2-cat$_2$ as control plasmids in which a 1.0- to 1.5-kb *Bam*HI DNA fragment without a (dT-dG)$_n$, randomly isolated from the human genome, was inserted at the same site.

CV-1 cells were transfected with these DNAs by $CaPO_4$-mediated transfection, and expression was monitored by assay of CAT activity after 48 h (Fig. 2). In this experiment, 23% of the chloramphenicol was converted to the acetylated form by the extract of cells transfected with pSV2-cat DNA. pSV2-cat$_1$ and pSV2-cat$_2$ DNAs produced about the same or slightly less CAT activity than did control pSV2-cat DNA. In contrast, all five constructs containing the TG-element showed increased CAT activity (2.5 to 4 times that of pSV2-cat). Southern blotting analysis of DNA from the transfected cells indicated that there was no significant difference in the amount of DNA incorporated (data not shown).

Although pSV2-cat DNA cannot replicate in CV-1 cells, it can replicate in COS-1 cells, a cell line derived from CV-1 by transformation with an origin-defective SV40 (4). An advantage of using the COS-1 cells recipients is that all the replicated DNA should be packaged into minichromosomes, whereas the structure of the unreplicated DNA in CV-1 cells is uncertain. To determine whether increased expression can be observed with the replicated DNA, pSV2-cat, pSV2-cat$_{3\text{-}2}$ and pSV2-cat$_{3\text{-}7}$ DNAs were transfected to COS-1 cells, and then the CAT activity and the amount of the replicated DNA were determined at 2 or 4 days after transfection. Again, pSV2-cat$_{3\text{-}2}$ and pSV2-cat$_{3\text{-}7}$ DNAs showed more (two- three-fold) CAT activity than did pSV2-cat DNA at both times (Fig. 3A). The increased CAT level directed by pSV2-cat$_{3\text{-}2}$ and pSV2-cat$_{3\text{-}7}$ DNAs relative to that directed by pSV2-cat DNA did not reflect differences in the amounts of these templates, since the amounts of replicated pSV2-cat$_{3\text{-}2}$ and pSV2-cat$_{3\text{-}7}$ DNAs were the same or even slightly less than that of pSV2-cat DNA (Fig. 3B).

(ii) In pA$_{10}$cat (enhancer minus). The pSV2-cat plasmid we used for the pSV2-cat derivatives in the previous experiment contains the SV40 72-bp repeats, which are known to be strong enhancer elements (2, 7). The expression of the CAT gene was therefore strongly dependent on the presence of this enhancer (5, 13). To examine whether these fragments can enhance the CAT gene expression independently of the SV40 enhancer, the same three genomic DNA fragments were next cloned in both orientations at a *Bgl*II site (the 5′ site of the CAT gene) or at a *Bam*HI site (the 3′ site of the CAT gene) of an enhancer-minus plasmid, pA$_{10}$-cat (Fig. 4). pA$_{10}$-cat has a structure similar to that of pSV2-cat except

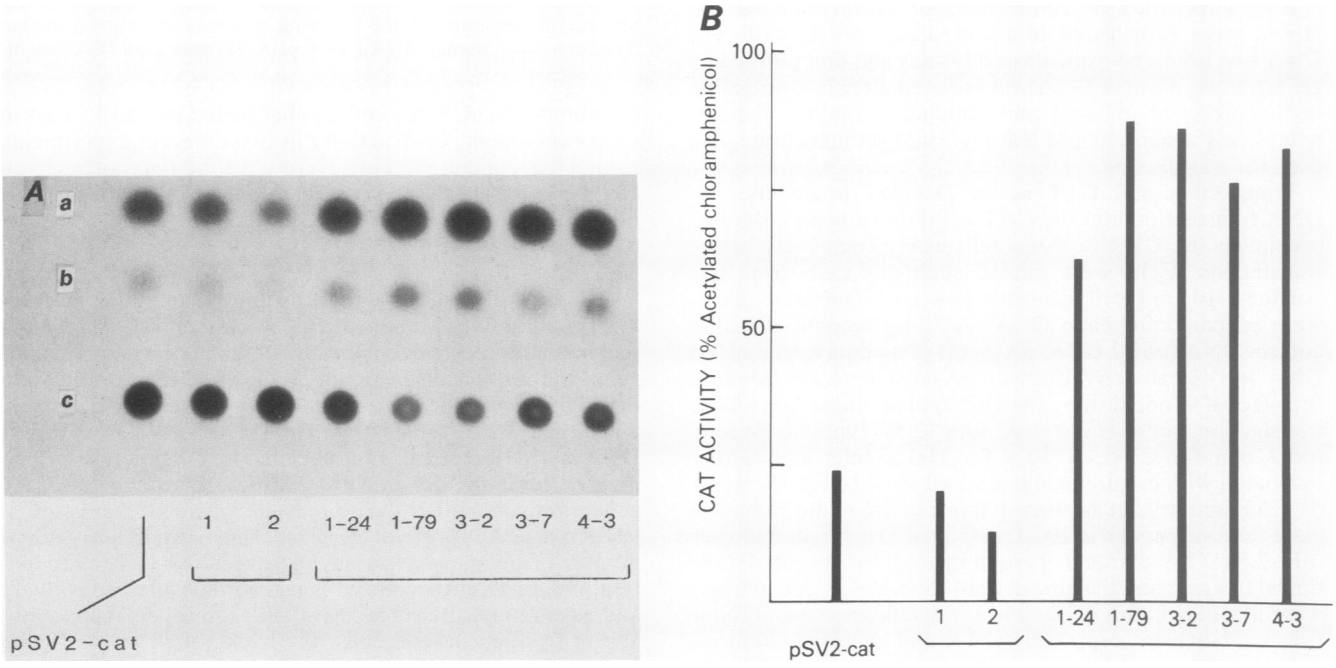

FIG. 2. CAT activity produced in CV-1 cells by the pSV2-cat and its derivatives. pSV2-cat, pSV2-cat$_{1,2}$, pSV2-cat$_{1\text{-}24}$, pSV2-cat$_{1\text{-}79}$, pSV2-cat$_{3\text{-}3}$, pSV2-cat$_{3\text{-}7}$, and pSV2-cat$_{4\text{-}3}$ were transfected to CV-1 cells. Cell extract (10 μl) was assayed for CAT activity. (A) Autoradiogram: spots *a* and *b* are both acetylated chloramphenicol, and spot *c* is unacetylated chloramphenicol. (B) Quantification of CAT activity: spots *a*, *b*, and *c* in A were cut, and the radioactivity was determined; CAT activity is shown as percent acetylated chloramphenicol (Percentage of $a + b/a + b + c$).

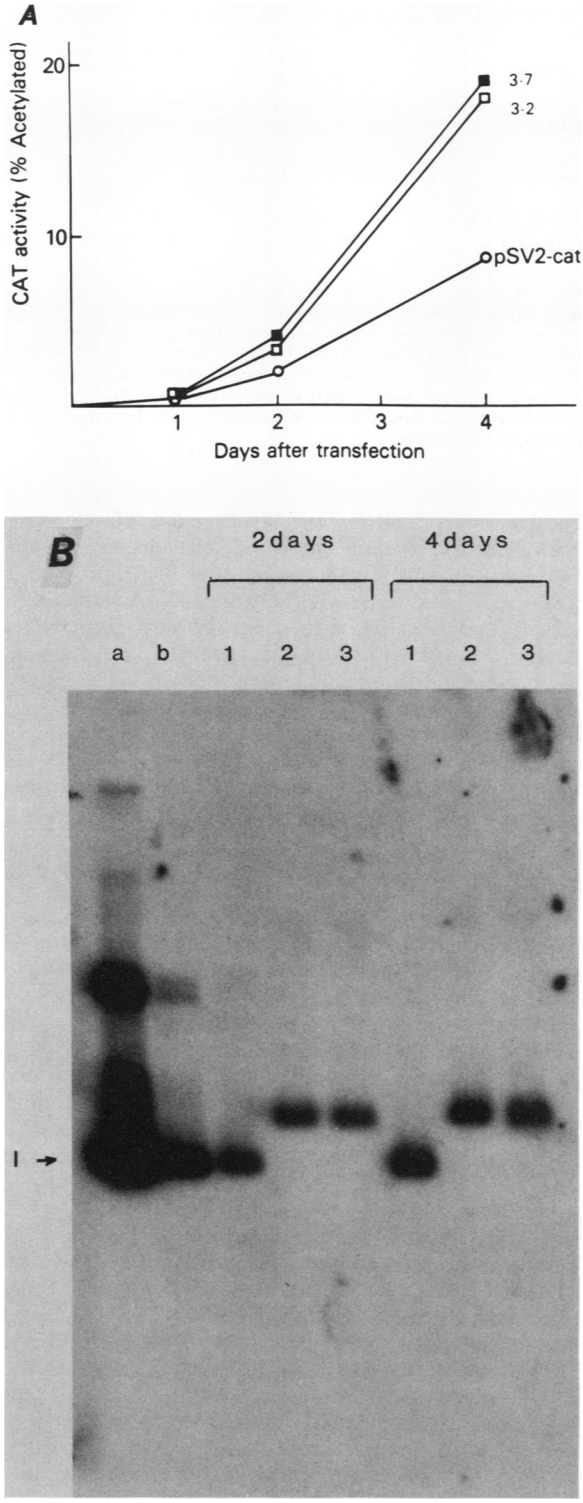

FIG. 3. CAT activity produced in COS-1 cells by the pSV2-cat and its derivatives. pSV2-cat, pSV2-cat$_{3-2}$, and pSV2-cat$_{3-7}$ were transfected to COS-1 cells. At 1, 2, or 4 days after transfection, cells were harvested and divided into two parts. One half was used for the CAT assay (A). The other half was used to determine the amount of the replicated DNA (B). For the latter analysis, total DNA was extracted from the cells, subjected to gel electrophoresis, blotted, and hybridized to nick-translated pSV2-cat DNA. The arrow indicates the position of form I pSV2-cat DNA. In lanes a and b, 10 and 1 ng of pSV2-cat DNA were applied, respectively.

that the SV40 72-bp repeats are deleted in pA$_{10}$-cat. pA$_{10}$-cat and its derivatives were transfected into CV-1 cells. Representative results obtained with a series of pA$_{10}$-cat plasmids with insertions of fragment 4 are shown in Fig. 5. The parental pA$_{10}$-cat DNA showed much less CAT activity than did pSV2-cat (1/200 that of pSV2-cat) because of the lack of the SV40 enhancer. However, the CAT activity produced by pA$_{10}$-cat was significantly higher than background (the extract from untransfected CV-1 cells showed no CAT activity). When fragment 4 was inserted at the 3′ site, plasmids (pA$_{10}$-3′-4a and pA$_{10}$-3′-4b) produced two to three times more CAT activity than did pA$_{10}$-cat DNA. The enhanced CAT activity was more pronounced in pA$_{10}$5′-4a and pA$_{10}$5′-4b DNAs, in which the fragment was placed at the 5′ site (10 to 15 times that of pA$_{10}$-cat DNA). The same level of enhancement was observed in two constructions with the TG-element in the opposite orientation (compare p3′-4a to p3′-4b, or p5′-4a to p5′-4b), indicating that the orientation of the fragment is not crucial to its enhancer activity. Similar enhancement was observed with two other DNA fragments (fragments 1 and 3) (data not shown). The enhancer activity of these genomic fragments was, however, less than that of the SV40 enhancer (about 1/10 to 1/20 that of the SV40 enhancer).

The same set of plasmids carrying fragment 4 was used to transfect HeLa cells to determine whether this DNA fragment is active in other cells of another species (Fig. 5). Fragment 4 increased the CAT activity 2 to 3 times when it was located at the 3′ site (p3′-4a and p3′-4b), whereas the

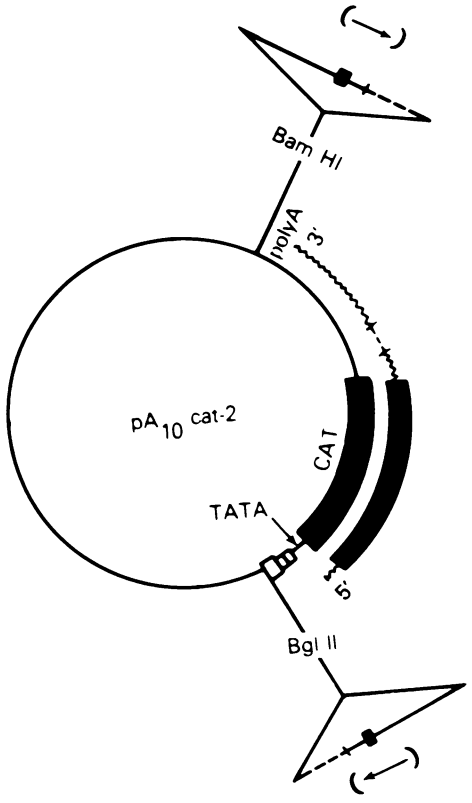

FIG. 4. Schematic diagram pA$_{10}$-cat (enhancer minus) and its derivatives. The construction of pA$_{10}$-cat2 has been described previously (13). A genomic fragment with a TG-element (fragments 1, 3, and 4) or a TG-element alone of variable length was inserted at a *Bam*HI site or a *Bgl*II site of pA$_{10}$-cat.

enhancement was 10 to 12 times when at the 5′ site (p5′-4a and p5′-4b). This $(dT\text{-}dG)_n$-containing fragment was, therefore, equally active both in monkey cells and in human cells, although, as in CV-1 cells, it was much less active than the SV40 enhancer in HeLa cells. However, fragment 4 was as active as the human DNA sequence homologous to BK virus that Rosenthal et al. (22) have isolated and shown to have an enhancer activity in HeLa cells (compare p5′-4a or p5′-4b to the BKV homologous sequence in Fig. 5).

Enhanced CAT gene expression by the TG-element alone. The results described above indicated that three DNA fragments with the TG-element contain some DNA sequence(s) with enhancer activity. Since these DNA fragments were randomly isolated from a human DNA library by hybridization to poly(dT-dG) · poly(dC-dA) and since the only common nucleotide sequence found in these fragments is the TG-element, it seemed likely that the enhanced expression was due to the TG-element. However, the possibility still remained that an enhancer-like sequence other than the TG-element was associated with the three fragments. To ensure that the TG-element itself has an enhancer activity, chemically synthesized poly(T-G) · poly(C-A), instead of the genomic DNA fragments, was cloned in pA_{10}-cat. Poly(dT-dG) · poly(dC-dA) of various lengths (50 to 130 bp) was inserted either at the 3′ site or at the 5′ site of pA_{10}-cat. These plasmids with a TG-element were transfected into CV-1 and HeLa cells, and the CAT activities induced by them were compared with that induced by the control plasmid pA_{10}-cat. Representative results obtained with the plasmids containing a 50- or 60-bp $(dT\text{-}DdG)_n \cdot (dC\text{-}dA)_n$ tract are shown in Fig. 6. The plasmids with a TG-element at the 3′ site (p3′-50, p3′-60, and p3′-70) produced two to four times more CAT activity than did $p5_{10}$-cat, both in CV-1 cells and in HeLa cells. The plasmids in which a TG-element is located at the 5′ site (p5′-50a and p5′-50b) induced 7 to 9 and 8 to 10 times more CAT activity than did pA_{10}-cat in CV-1 cells and in HeLa cells, respectively. These results confirm the conclusion that the TG-element itself has enhancer activity. Morever, the TG-element alone is as active as genomic fragment 4 containing the TG-element, although fragment 4 was slightly more active than 50 bp of $(dT\text{-}dG)_n \cdot (dC\text{-}dA)_n$ alone (Table 1). The slight difference in enhancer activity between fragment 4 and 50 bp of $(T\text{-}G)_n \cdot (C\text{-}A)_n$ is probably due to the difference in size of the TG-elements in the two plasmids [fragment 4 contains a $(T\text{-}G)_{20} \cdot (C\text{-}A)_{20}$]. Enhancer activities of various lengths of $(T\text{-}G)_n \cdot (C\text{-}A)_n$ are compared in Fig. 7. $(T\text{-}G)_n \cdot (C\text{-}A)_n$ (50 bp) alone and fragment 3 containing 50 bp of $(T\text{-}G)_n \cdot (C\text{-}A)_n$ showed the same level of enhancement, which was slightly lower than that of fragment 4. The maximum enhancement was obtained with 30 to 40 bp of $(T\text{-}G)_n \cdot (C\text{-}A)_n$, although the activity of shorter $(T\text{-}G)_n \cdot (C\text{-}A)_n$ has not been determined. As the length increased from 40 to 130 bp, the enhancer activity fell. A length of 130 bp of $(T\text{-}G)_n \cdot (C\text{-}A)_n$, for example, was fivefold less active than 50 bp of $(T\text{-}G)_n \cdot (C\text{-}A)_n$. Interestingly, the size of the major TG-element found in the genome is between 20 and 60 bp, which is the size that seems to have maximum enhancer activity in this system. These results indicate that all, or a major part, of the enhancement observed with the genomic fragments (Fig. 2, 3, and 5) is due to the enhancer activity of the TG-element.

Another sequence with Z-DNA potential does not enhance CAT gene expression. Sequences with alternating purines and pyrimidines such as the TG-element can be in the left-handed conformation (Z-DNA) when in appropriately supercoiled plasmids (11, 18). Since Z-DNA has been found in viral enhancers, it is possible that the TG-element enhances gene expression simply because it can assume the Z-conformation and, furthermore, that it is active when in the Z-form. To examine whether other DNA sequences will have enhancer activity if they can be in Z-form, we constructed a derivative of pA_{10}-cat with a $(dG\text{-}dC)_{15} \cdot (dG\text{-}dC)_{15}$ insert. This sequence has been shown to have a greater probability of being in the Z-form than the $(dT\text{-}dG)_n \cdot (dC\text{-}dA)_n$ sequence (16, 21). Thus, if the simple presence of a sequence with Z-form potential enhances gene expression, then the $(dG\text{-}dC)_n \cdot (dG\text{-}dC)_n$ sequence should be at least as active as $(dT\text{-}dG)_n \cdot (dC\text{-}dA)_n$, possibly more so. CAT activity assay results with extracts of HeLa cells infected with pA_{10}-cat, pA_{10}-cat with the TG-element (p5′-50a), and pA_{10}-cat with $(dG\text{-}dC)_{15} \cdot (dG\text{-}dC)_{15}$ at the 5′ site are shown (Fig. 8). As before, p5′-50a showed about eightfold more CAT activity than did the control plasmid. However, in contrast to the prediction of the preceding argument, the $(dG\text{-}dC)_{15} \cdot (dG\text{-}dC)_{15}$ insert did not enhance CAT gene expression. The level of CAT activity was actually equivalent to that in uninfected cells, suggesting that the $(dG\text{-}dC)_{15} \cdot (dG\text{-}dC)_{15}$ sequence rather inhibited the CAT gene expression. We have also observed the inhibitory effect of the $(dG\text{-}dC)_{15} \cdot (dG\text{-}dC)_{15}$ insert in the pSV2-cat plasmid. Similar results were obtained when CV-1 cells were transfected.

TABLE 1. Relative enhancer activity of the TG-element[a]

Plasmid	Location of TG-element	CAT gene expression in:	
		CV-1	HeLa
pSV2-cat		200	500
$pA_{10}2$-cat		1	1
Genomic DNA (fragment 4)	3′ site	2–3	3–4
	5′ site	13–15	14–16
$(T\text{-}G)_n \cdot (C\text{-}A)_n$ ($n = 25$)	3′ site	3	4
	5′ site	7–9	8–10

[a] The levels of CAT gene expression directed by pSV2-cat (SV40 enhancer positive) and pA_{10}-cat derivatives with the TG-element are compared with that by pA_{10}-cat (enhancer minus) in each cell type. The average values obtained from two or three different experiments (like those shown in Fig. 5 and 6) are summarized here.

DISCUSSION

The results presented here indicate that the TG-element is capable of enhancing gene expression in vivo. Since in previous studies the level of CAT activity has been found to correlate with the amount of CAT mRNA (5), it is likely that the TG-element increases the level of CAT gene transcription. The properties of the TG-element are similar to those of viral enhancers: the activity is not strictly dependent on either orientation or position relative to the gene (although both the TG-element and viral enhancers are more effective when closer to the promotor). These similarities suggest that the TG-element may function as a transcriptional enhancer.

The enhancer activity of the TG-element was considerably lower than that of the SV40 enhancer in the pA_{10}-cat system. A rationale for the strength of viral enhancers has been offered in the suggestion that viruses have evolved to compete effectively with transcriptional units in the host cells. The relatively low enhancing activity of the TG-element may be typical of some other cellular enhancers. The human enhancer sequence which is homologous to the BK

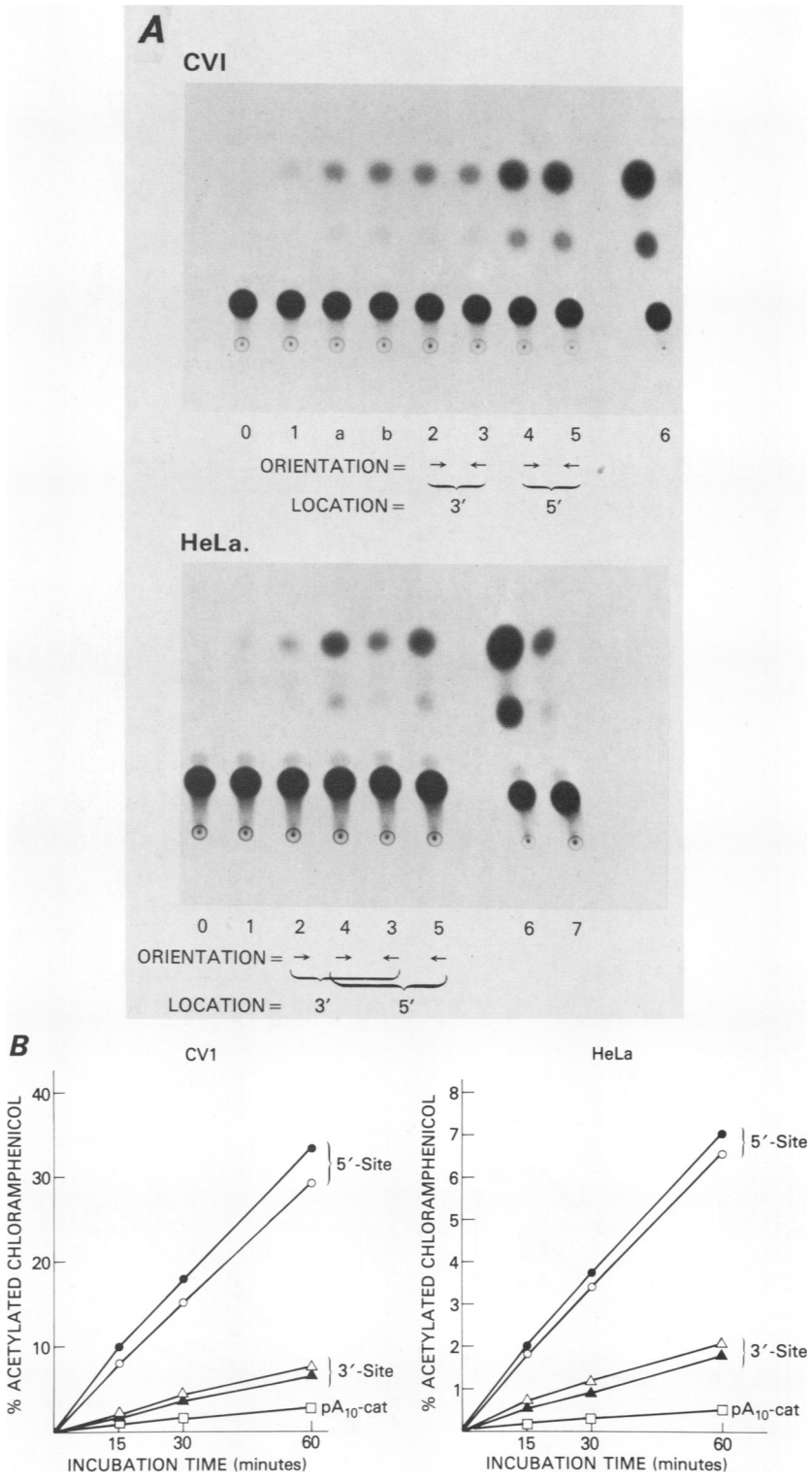

FIG. 5. Enhanced CAT gene expression by fragment 4 in an enhancer-minus plasmid. pA_{10}-cat (lane 1), p3′-4a (lane 2), p3′-4b (lane 3), p5′-4a (lane 4), p5′-4b (lane 5), p3′-3a (lane a), p3′-3b (lane b), pSV2-cat (lane 6), and pA_{10}-cat with BKV-homologous human sequence at the *Bgl*II site (lane 7) (22) were transfected into either CV-1 cells or HeLa cells. (A) Autoradiogram of CAT activity. A 30-μl volume of each cell extract (except 6 μl of cell extract was used for pSV2-cat) was incubated for 30 min. In lane 0 untransfected CV-1 or HeLa cells were used. (B) Kinetic analysis of CAT activity. The same cell extract was incubated for 15, 30, or 60 min. Symbols: □, pA_{10}-cat; △, p3′-4a; ▲, p3′-4b; ○, p5′-4a; ●, p5′-4b.

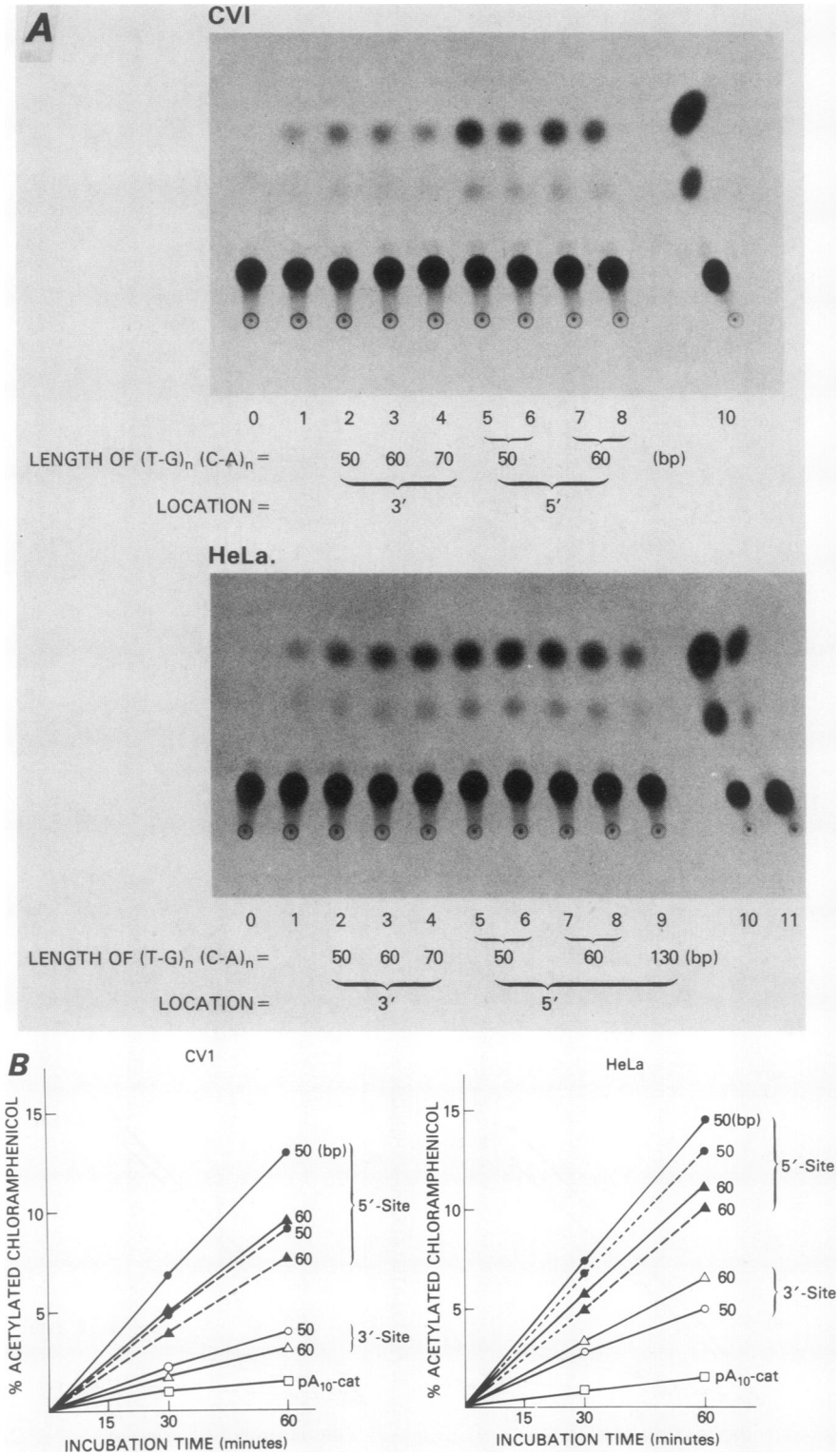

FIG. 6. Enhanced CAT gene expression by the TG-element alone. pA_{10}-cat (lane 1), p3′-50 (lane 2), p3′-60 (lane 3), p3′-70 (lane 4), p5′-50a (lane 5), p5′-50b (lane 6), p5′-60a (lane 7), p5′-60b (lane 8), p5′-130 (lane 9), pSV2-cat (lane 10), and pA_{10}-cat with the BKV-homologous sequence (11) were transfected into either CV-1 cells or HeLa cells. (A) Autoradiogram of CAT assay. A 30-μl volume of each cell extract (except 6 μl for pSV2-cat) was incubated for 30 min. Lane 0 shows the CAT activity in untransfected cells. (B) Kinetic analysis of CAT activity. The same extracts were incubated for 15, 30, or 60 min. Symbols □—□, pA_{10}-cat; ○—○, p3′-50; △—△, p3′-60; ●—●, p5′-50a; ●---●, p5′-50b; ▲—▲, p5′-60a; ▲---▲, p5′-60b.

viral enhancer has about the same activity in the pA_{10}-cat assay system as does the TG-element. However, a relatively strong cellular enhancer has been identified in immunoglobulin genes (1, 3), and it seems likely that different cellular enhancers will have a wide range of strengths. Although the TG-element may not be a particularly strong enhancer, it has one property which distinguishes it from all known enhancer sequences: it is a moderately repeated sequence in many (probably all) eucaryotic genomes. The human genome, for example, has ca. 10^5 copies, which is roughly equivalent to one copy per gene. Another feature of interest is that it was equally active in monkey and human cells. This is in contrast to the behavior of the viral (14) and cellular (found in the immunoglobulin gene [1, 3]) enhancers described previously, which show species or tissue, or both, specificity. Considering the widespread distribution of the sequence, it is likely that the TG-element would be active in many eucaryotic cells.

The mechanism of enhancement by the TG-element, or, for that matter, enhancers generally, is not understood at this time. The observation that potential Z-DNA sequences are found in the SV40 enhancer and many other viral enhancers (19) has provoked considerable interest in the possible relationship between Z-DNA and enhancer function. Although this is an intriguing possibility, our results with pA_{10}-cat-GC plasmid suggest that the simple presence of a sequence with the potential for the Z-conformation is insufficient for enhancement. If the Z-form of the TG-element is necessary for enhancer function, then the inactivity of the $(dG\text{-}dC)_n \cdot (dG\text{-}dC)_n$ insert might be due to critical structural differences between the two sequences such that only the TG-element interacts productively with appropriate cell factors which are necessary for transcription. A class of proteins that specifically bind to Z-DNA have been found in *Drosophila* nuclei, and they seemed to have a higher affinity to the Z-form of $(dT\text{-}dG)_n \cdot (dC\text{-}dA)_n$ than to the Z-form of $(dG\text{-}dC)_n \cdot (dG\text{-}dC)_n$ (20). It is also possible that the TG-element has yet another non-B-form, structure in vivo which distinguishes it from both B-DNA and the Z-form of $(dG\text{-}dC)_n \cdot (dG\text{-}dC)_n$, and which underlies its activity. A simple poly(purine) · poly(pyrimidine) sequence found in promotor regions of chicken globin gene (17, 23) is shown to be able to adopt a non-B but non-Z conformation. These possibilities cannot be distinguished at this time, since the structure of these elements on the actual transcription template in vivo is not known. Studies on the S1 nuclease sensitivity of the TG-element have shown that the TG-element is sensitive to cleavage when in supercoiled plasmids in vitro but not detectably sensitive when in minichromosomes in isolated nuclei (10).

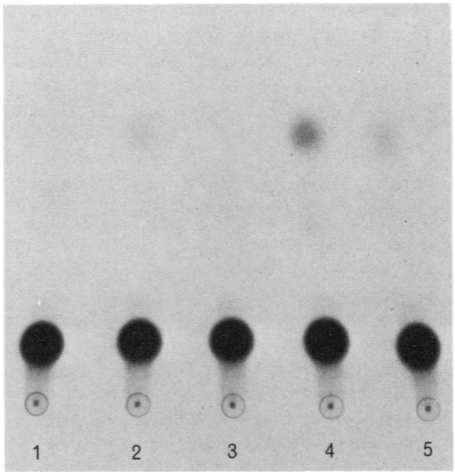

FIG. 8. Activity of pA_{10}-cat(GC). $(dG\text{-}dC)_n \cdot (dG\text{-}dC)_n$ ($n = 15$) was inserted in pA_{10}-cat at the *Bgl*II site (5′ to the CAT gene). This construction [pA_{10}-cat(GC)] was used to transfect HeLa cells. Lanes: 1, no DNA; 2 and 5, two different preparations of pA_{10}-cat; 3, pA_{10}-cat(GC); 4, p5′-50a [with $(T\text{-}G)_n \cdot (C\text{-}A)_n$, $n = 25$].

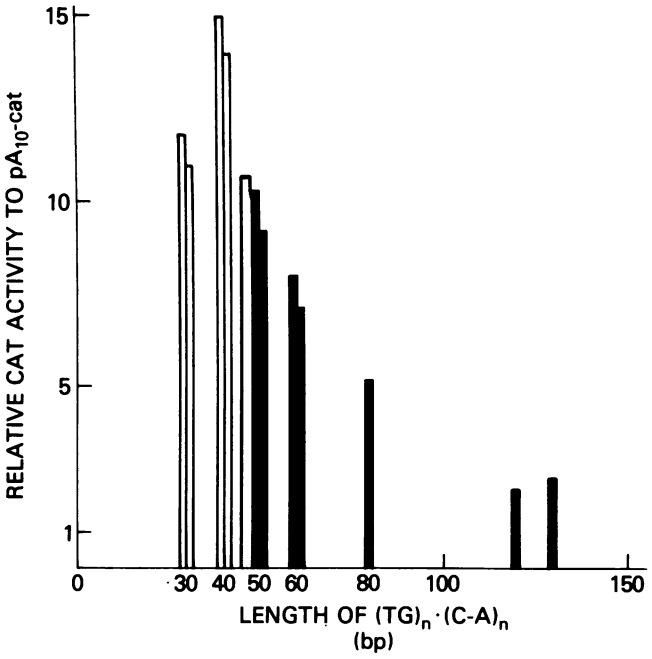

FIG. 7. Effect of the length of $(T\text{-}G)_n \cdot (C\text{-}A)_n$ on its enhancer activity. Enhancing activities of the three genomic fragments and of synthetic TG-elements of various lengths are shown. pA_{10}-cat derivatives containing these DNAs at the 5′ site were transfected into HeLa cells. CAT activity produced by each plasmid was compared to that produced by control pA_{10}-cat. Open bars indicate the enhancing activity observed with the genomic fragments. Solid bars show the enhancing activity of $(T\text{-}G)_n \cdot (C\text{-}A)_n$ alone. Actual plasmids used here were plotted at: 30 bp, p5′-1a and p5′-1b; 40 bp, p5′-4a and p5′-4b; 50 bp, p5′-3a and p5′-3b; 50 bp, p5′-50a and p5′-50b; 60 bp, p5′-60a and b; 80 bp, p5′-80; 120 bp, p5′-120; and 130 bp, p5′-130.

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