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Selection of DNA clones with enhancer sequences

(DNA replication/gene transcription/polyoma DNA)

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ABSTRACT **A method is described for selection of DNA clones that contain enhancer sequences that activate gene expression. An *Escherichia coli*-rodent cell shuttle vector, pPyE0, was used that contains polyoma viral DNA without the polyoma enhancer region. Replication of pPyE0 DNA in mouse cells is markedly reduced due to deletion of the polyoma enhancer region. Insertion of mouse genomic DNA fragments that contain putative enhancer sequences into pPyE0 adjacent to the polyoma origin of replication restored, to varying extents, the ability of the recombinant plasmid DNA to replicate in mouse cells. Recombinant plasmids that replicate well in mouse cells, therefore, are amplified selectively. Transfection of mouse neuroblastoma or fibroblast cells that constitutively synthesize polyoma large tumor antigen with a library of mouse genomic DNA fragments inserted in pPyE0 yielded many recombinant plasmids. DNA inserts from each of the 16 clones that were examined stimulated the expression of an enhancerless chloramphenicol acetyltransferase reporter gene. The DNA inserts from 4 clones that were studied resulted in 4- to 13-fold increases in chloramphenicol acetyltransferase mRNA in transfected mouse cells. Nucleotide sequence analysis led to the identification of 5 genomic DNA clones that were obtained by selection. All of the homologies found were to regions of DNA that are thought to be involved in the regulation of gene expression.**

Replication of polyoma viral DNA requires three viral factors in addition to cellular factors, the polyoma DNA sequence that functions as the *ori* (1), polyoma large tumor (T) antigen encoded by the polyoma early gene (2), and the enhancer region of polyoma DNA that binds enhancer proteins that activate viral DNA replication and gene transcription (3). Polyoma large T antigen binds to multiple sites at the polyoma *ori* (4), has ATP phosphohydrolase activity (5), and probably functions as a DNA helicase.

Most enhancer sequences have been found by demonstrating that nucleotide sequences in DNA are binding sites for proteins that stimulate the initiation of gene transcription in an orientation- and position-independent manner (6). Other experimental approaches also have been used to isolate cellular enhancers. For example, the integration of enhancerless polyoma DNA into rat genomic DNA was used to obtain transformed cells that were highly tumorigenic due to rat genomic DNA enhancers that increased the expression of the polyoma large T antigen gene (7). Monkey cells were cotransfected with linear, enhancerless simian virus 40 (SV40) DNA and either DNA fragments from other viruses that contain enhancers or cellular DNA fragments, in an attempt to generate replicating viruses with enhancers that activate transcription of the large T-antigen gene (8). Cells were transfected with DNA from an enhancerless SV40 vector ligated with bovine papilloma, Molony murine sarcoma, or hepatitis B viral DNA fragments, and replicating recombinant plasmids were recovered that contained DNA inserts with viral enhancers (9, 10). In addition, cells were stably transformed by plasmid DNA that contained an enhancerless SV40 promoter linked to xanthine-guanosine phosphoribosyltransferase gene (11) or a neomycin-resistance gene (12). Enhancers in cellular DNA near sites of integration of plasmid DNA activate these genes and confer upon stably transformed cells resistance to mycophenolic acid or G418, respectively.

In this report a method is described for selection of DNA clones that contain enhancer sequences that activate replication of an enhancerless polyoma vector.‡

MATERIALS AND METHODS

Bacterial Strains, Cell Lines, and Media. *Escherichia coli* NM554 cells (13) were transformed with a pPyE0-mouse genomic DNA library. *E. coli* DH5αMCR cells (GIBCO BRL) were transformed by recombinant DNA obtained by selection in mouse cells. N18TG-2 mouse neuroblastoma (14) and mouse WOP 3027-3 fibroblasts (15) (a gift from C. Basilico) were grown in the first formulation of Dulbecco's modified Eagle's medium (GIBCO BRL catalog no. 430-2100) and 10% fetal bovine serum at 37°C in 10% CO_2/90% air.

Plasmids. Shuttle vector pPyE0 was constructed by inserting an oligodeoxynucleotide containing a T7 RNA polymerase promoter, a *Bgl* II site, and a *Xho* I site into pPyPBΔ (16), a gift from R. Kamen, which consists of polyoma DNA minus the enhancer region, with pAT153 DNA inserted into the polyoma *Bam*HI site (Fig. 1). pPy-1Δ*ori*, which cannot replicate in mouse cells, was constructed from pPy-1 (p53.A6.6) (17) DNA, which contains pAT153AvaI$^-$ DNA inserted in the polyoma *Bam*HI site, by excision of part of the polyoma *ori* with *Apa* I and *Nar* I [nucleotide residues 7–87 according to the numbering system of Soeda *et al.* (18)]. pPy-1CAT (used to normalize replication of plasmids in mouse cells), which contains a *cat* gene that confers chloramphenicol resistance on *E. coli* cells, was constructed as follows: pPy-1 DNA was cleaved within the β-lactamase gene by *Pvu* I, treated with T4 DNA polymerase, and ligated in the correct orientation with the T4 DNA polymerase-treated *Sal* I fragment of pCM1 (Pharmacia) containing the Tn*9 cat* gene. To construct pA10CAT2KX, an enhancerless chloramphenicol acetyltransferase (CAT) reporter vector, pA10CAT2 (19) (a gift from B. H. Howard), was modified by insertion of an oligodeoxynucleotide containing a *Kpn* I and a *Xho* I site into the *Bgl* II site of the vector.

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Abbreviations: CAT, chloramphenicol acetyltransferase; IAP, intracisternal A particle; SV40, simian virus 40; LTR, long terminal repeat.

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‡The sequences reported in this paper have been deposited in the GenBank data base [N1, W4, W8, W13, and W20 (accession nos. U08126, U08129, U08130, U08127, and U08128, respectively)].

Mouse Genomic DNA Library. Mouse genomic DNA was prepared from liver, digested partially with *Mbo* I, and fractionated by agarose gel electrophoresis. The 2- to 4-kb DNA fraction, which also contained some smaller DNA fragments, was ligated with pPyE0 DNA that had been cleaved by *Bgl* II and the DNA was introduced into *E. coli* NM554 cells. Four mouse genomic DNA libraries were prepared; the pooled library contained 9.4×10^5 recombinants.

Transfection Procedure. Plasmid DNA in cleared *E. coli* lysates (alkaline/SDS lysis method) was purified by two cesium chloride centrifugations and deproteinized. Mouse cells were transfected with plasmid DNA by the calcium phosphate coprecipitation method (20). Cells were plated in 100-mm Petri dishes (5×10^5 per dish for N18TG-2T or 2×10^5 per dish for WOP cells) and transfected the next day with 20 μg of plasmid DNA per dish. The transfection period was 4 hr for N18TG-2T cells and 18 hr for WOP cells. Cells were subjected to a glycerol shock, the medium was replaced with fresh medium and serum, and the cells were incubated for 2 or 3 days in 10% CO_2/90% air.

Construction of N18TG-2T. To construct N18TG-2T mouse neuroblastoma cells that constitutively synthesize the polyoma large T antigen, pPy-1Δ*ori* and pRSV*neo* (a gift from B. H. Howard) were cotransfected into N18TG-2 cells. Stable transformants resistant to G418 (GIBCO) were selected and polyoma large T-antigen levels were determined by indirect immunofluorescence. N18TG-2T then was cloned.

Assay of CAT and β-Galactosidase Activities. Transfected cells were harvested and lysed, and CAT activity was determined by standard methods (21, 22). β-Galactosidase (EC 3.2.1.23) activity was assayed using *o*-nitrophenyl β-galactopyranoside (23).

DNA Sequencing. The nucleotide sequences of both strands of DNA were determined by the dideoxynucleotide chain termination method (24). Sequences were analyzed using University of Wisconsin Computer Group programs.

CAT mRNA Analysis. A DNA clone for synthesis of an RNA probe for CAT mRNA (probe 1) was constructed by subcloning the *Bgl* II–*Pvu* II fragment of pA10CAT2 DNA (Fig. 3*A*) into *Bgl* II–*Pvu* II-treated pSP71 (Promega). A DNA clone for synthesis of an RNA probe for β-galactosidase mRNA (probe 2) was prepared from pCH110 by insertion of an oligonucleotide with a T7 RNA promoter sequence into the *Kpn* I site of pCH110; the plasmid was linearized with *Hin*dIII. Probes were labeled by incorporation of [α-^{32}P]CTP into RNA catalyzed by T7 RNA polymerase. RNA was subjected to PAGE (5% polyacrylamide gels containing 8 M urea).

RESULTS

Enhancer Selection Strategy. pPyE0, shown in Fig. 1, is a rodent cell-*E. coli* shuttle vector that consists of polyoma A2 DNA minus the viral enhancer region (nucleotide residues 5027–5269); an oligonucleotide containing a T7 RNA polymerase promoter, a *Bgl* II cloning site, and a *Xho* I site was inserted at the site of the deleted viral enhancer region; and pAT153 DNA (derived from pBR322) containing an *ori* that functions in *E. coli* and a constitutive β-lactamase gene was inserted in the late polyoma gene. pPyE0 replicates poorly in mouse cells because the enhancers that activate polyoma DNA synthesis have been deleted. Mouse genomic DNA fragments, partially digested with *Mbo* I, were inserted in the *Bgl* II site, thereby replacing the polyoma enhancer region. Hence plasmids with mouse genomic DNA inserts that contain enhancers that activate plasmid replication were expected to be amplified selectively.

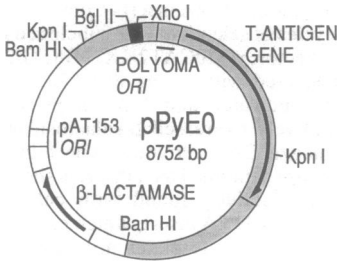

FIG. 1. pPyEO, an enhancerless polyoma *ori* reporter shuttle vector.

Recombinant plasmids used to transfect mouse cells were obtained from transformed *E. coli* NM554, which expresses the Dam DNA methylase that catalyzes methylation of the N^6 amino group of the dA residue in GATC. No DNA methylase with this specificity has been detected in mouse cells. Hence, recombinant DNA molecules that replicate in mouse cells would contain dA residues in GATC that are not methylated. Mouse cells were transfected with methylated recombinant DNA and incubated for several days. Then plasmid DNA was harvested from transfected cells and incubated with *Dpn* I, which cleaves GATC sites in DNA that are methylated on both strands (such as plasmid DNA synthesized in *E. coli*) but does not cleave DNA at hemimethylated or unmethylated GATC sites (such as plasmid DNA synthesized in mouse cells). Plasmid DNA that was not destroyed by *Dpn* I was used to transform *E. coli* DH5αMCR cells, which also express the Dam DNA methylase, and ampicillin-resistant *E. coli* cells were obtained. Plasmid DNA was harvested, purified, and the entire cycle of selection was repeated. The number of recombinants recovered was increased by the use of N18TG-2T and WOP mouse cells, which constitutively synthesize polyoma large T antigen (not shown).

Selection of DNA Clones with Enhancers that Activate Plasmid Replication. The results of three selection experiments for recombinants with mouse genomic DNA inserts that activate replication of pPyE0 in N18TG-2T mouse neuroblastoma cells (experiments 1 and 2) or WOP mouse fibroblast cells (experiment 3) are shown in Table 1.

Table 1. DNA clones obtained by selection

Exp.	Plasmids recovered	Clones found		% of clones	Size of DNA insert, bp
		Kind	No.		
1	≈100,000	N1	43/50	86	163
		N5	5	10	2640
		N13	1	2	2600
		N25	1	2	2800
2	≈100,000	N106	13/49	26	413
		N108	5	10	300
		N124	4	8	2600
		N136	3	6	2200
		N131	2	4	2200
		Other*	1 each	2 each	
3	≈30,000	W6	10/24	42	2200
		W8	5	21	123
		W4	2	8	386
		W2	1	4	3400
		W5	1	4	3000
		W11	1	4	2200
		W13	1	4	188
		W14	1	4	2300
		W20	1	4	457
		W21	1	4	2500

The results of three selection experiments are shown: selection of pPyEO recombinants with mouse genomic DNA inserts that activate plasmid DNA replication in mouse neuroblastoma N18TG-2T (experiments 1 and 2) and mouse fibroblast WOP cells (experiment 3). *Twenty-two other clones.

N18TG-2T and WOP cells were transfected separately with a mouse genomic DNA library in pPyE0 (9.4×10^5 recombinants). Two cycles of selection were performed for each experiment. Approximately 100,000, 100,000, and 30,000 recombinants were recovered in experiments 1, 2, and 3, respectively. Fifty, 49, and 24 recombinant clones were picked randomly from the recombinants recovered in experiments 1, 2, and 3, respectively, and DNA from each clone was subjected to restriction site analysis with *Kpn* I, *Xho* I, *Sau*3AI, or a mixture of *Kpn* I and *Xho* I. The patterns of DNA fragments found provided a preliminary means of distinguishing different types of clones from multiple isolates of the same clone. Only four kinds of clones were found in experiment 1, 27 in experiment 2, and 10 in experiment 3. Eighty-six percent of the 50 clones characterized in experiment 1 were identified as clone N1 and 10% as N5. Multiple isolates were found for 5 clones in experiment 2 and for 3 clones in experiment 3.

Replication of Recombinant Plasmids. The ability of different cloned mouse genomic DNA inserts to stimulate plasmid replication is shown in Table 2. N18TG-2T or WOP cells were cotransfected with DNA from a recombinant plasmid that had been obtained by selection and Py-1CAT DNA used for normalization of plasmid replication values. pPyE0 recombinants contain a β-lactamase gene, which is constitutively expressed in *E. coli*; transformed *E. coli* cells therefore are resistant to ampicillin. pPy-1CAT contains the intact polyoma enhancer region and a *cat* gene inserted in the β-lactamase gene so that CAT is constitutively expressed in *E. coli* instead of β-lactamase. Thus, *E. coli* cells transformed with pPy-1CAT DNA are resistant to chloramphenicol rather than ampicillin. The transfected mouse cells were incubated for several days, and plasmid DNA was recovered and incubated with *Dpn* I. *E. coli* cells then was transformed with the *Dpn* I-resistant DNA, and the number of ampicillin-resistant and chloramphenicol-resistant colonies was determined separately. The fold increase in ampicillin-resistant *E. coli* colonies, normalized to a constant number of chloramphenicol-resistant colonies, is shown in the last column in Table 2. Replication of pPy-1, which contains the complete polyoma enhancer region, was 126- and 58-fold higher in N18TG-2T and WOP cells, respectively, than replication of pPyE0, which lacks the polyoma enhancer region. Three of the 10 recombinant clones examined with mouse genomic DNA inserts (W8, N1, and W21) stimulated plasmid replication markedly (9- to 86-fold) and 5 additional clones stimulated plasmid replication slightly (1.1- to to 1.6-fold), whereas no stimulation of plasmid replication was detected with 2 clones (N5 and N25). The apparent lack of effect of N5 DNA on plasmid replication was unexpected because 5 of the 50 recombinant clones that were examined in experiment 1 (Table 1) were N5 clones.

Table 2. Replication of recombinant plasmids

DNA used for cotransfection of mouse cells	No. of *E. coli* colonies		Fold increase in normalized Amp^R colonies
	Amp^R	Cm^R	
N18TG-2T cells			
pPyEO + pPy-1CAT	19,500	770,000	1.0
pPy-1 + pPy-1CAT	1,050,000	330,000	126.0
N1 + pPy-1CAT	440,000	860,000	20.0
N5 + pPy-1CAT	19,300	980,000	0.8
N13 + pPy-1CAT	37,500	925,000	1.6
N25 + pPy-1CAT	29,500	1,240,000	0.9
WOP cells			
pPyEO + pPy-1CAT	500	7,600	1.0
pPy-1 + pPy-1CAT	16,500	4,300	58.4
W2 + pPy-1CAT	500	7,000	1.1
W4 + pPy-1CAT	800	10,000	1.2
W8 + pPy-1CAT	53,000	9,400	85.7
W13 + pPy-1CAT	500	5,900	1.3
W20 + pPy-1CAT	800	7,800	1.6
W21 + pPy-1CAT	3,000	4,900	9.3

E. coli cells transformed with PyEO, pPy-1, or N or W series recombinant DNA express β-lactamase constitutively; therefore, these cells are resistant to ampicillin (Amp^R). pPy-1CAT contains the complete polyoma enhancer region and a *cat* gene that disrupts the β-lactamase gene; *E. coli* cells transformed with pPy-1CAT are resistant to chloramphenicol (Cm^R) rather than ampicillin. pPyEO is the shuttle vector that lacks the polyoma enhancer region; pPy-1 is similar to pPyEO but contains the polyoma enhancer region. The N and W series of clones consist of pPyEO with mouse genomic DNA inserts obtained by two rounds of selection in N18TG-2T or WOP cells, respectively (Table 1). N18TG-2T or WOP cells were cotransfected with 10 μg of the plasmid DNA indicated and 10 μg of pPy-1CAT DNA. Cells were incubated for 2 or 3 days, and plasmid DNA was obtained from the cells and incubated with *Dpn* I to cleave plasmid DNA that did not replicate in mouse cells. *E. coli* DH5αMCR cells then were transformed with *Dpn* I-resistant plasmid DNA and the number of Amp^R and Cm^R colonies was counted. Each value represents the average of values obtained in two experiments. The fold increase in Amp^R colonies due to the polyoma enhancer region (pPy-1) or mouse genomic DNA insert is shown in the last column; values for Amp^R colonies were normalized to a constant number of Cm^R colonies obtained with pPyEO + pPy-1 CAT DNA (770,000 for N18TG-2T or 7600 for WOP cells).

Effect of Cloned DNA on *cat* Gene Expression. The mouse genomic DNA inserts obtained by two cycles of selection were subcloned in the 5′ upstream region of an enhancerless *cat* reporter gene in pA10CAT2KX, which contains the SV40 early promoter (6 SP-1 sites and a TATA box) followed by the *cat* gene. DNA from each subclone was cotransfected into N18TG-2T or WOP cells with pCH110 DNA, which contains a constitutively expressed β-galactosidase gene used for normalization of CAT values. DNA from each of the 16 constructs of mouse genomic DNA ligated to the CAT reporter gene stimulated expression of the *cat* gene (2- to 30-fold increases in CAT activity compared to the vector alone) (Fig. 2). These DNA inserts also stimulated *cat* gene expression in mouse myoblast and rat glioma cells (data not shown). Stimulation of *cat* gene expression did not depend on the presence of polyoma large T antigen (not shown).

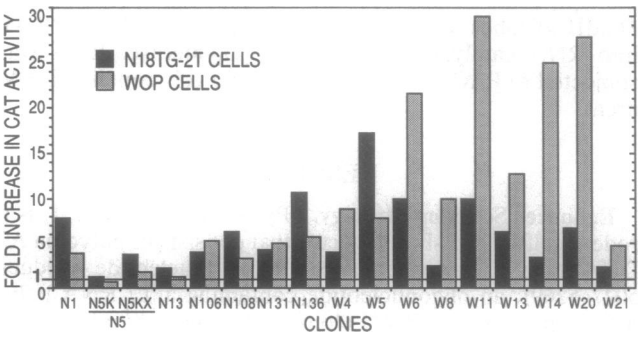

FIG. 2. Mouse genomic DNA inserts from 16 recombinant clones obtained by two cycles of selection were subcloned into an enhancerless CAT reporter vector, pA10CAT2KX. N18TG-2T cells and WOP cells were cotransfected with 13 μg of pA10CAT2KX-mouse genomic DNA clone indicated and 7 μg of pCH110 DNA, which encodes a constitutively expressed β-galactosidase whose activity was used to normalize CAT activities. The fold increase in CAT activity due to a mouse genomic DNA insert is shown on the ordinate relative to the CAT activity found with cells transfected with pA10CAT2KX without a DNA insert, which corresponds to 1. Mean values obtained with duplicate plates are shown. N5 contains an internal *Kpn* I site that was cleaved when N5 was excised from pPyEO. N5K is the 5′ portion, and N5KX is the 3′ portion of N5 adjacent to the polyoma *ori*.

CAT mRNA. CAT mRNA initiation sites and levels of CAT mRNA were determined with four mouse genomic DNA-pA10CAT2KX constructs, -N1, -W8, -W13, and -W20, or the vector alone, by means of RNase T1 protection experiments (Fig. 3). DNA from each *cat* reporter gene construct or the pA10CAT2KX vector without a mouse genomic DNA insert was cotransfected with pRAS-βGAL DNA (27), which contains a constitutively expressed chimeric *gpt/trpS/lacZ* gene, into N18TG-2T or WOP cells. Cells also were subjected to the transfection procedure without the addition of DNA. The cells were incubated for 2–3 days, total RNA was recovered, and portions of the RNA were hybridized with RNA probe 1 to determine CAT mRNA levels and initiation sites or with RNA probe 2 to determine levels of chimeric *gpt/trpS/lacZ* mRNA. Samples then were incubated with RNase T1 to digest single-stranded RNA. The region of probe 1 protected from RNase T1 by hybridization to CAT mRNA was expected to be 210, 214, and 215 nucleotides in length due to the SV40 early promoter (28). The protected region expected with probe 2 for the β-galactosidase mRNA transcripts was 210 nucleotide residues. As shown in Fig. 3*B*, CAT mRNA synthesis in N18TG-2T cells transfected by DNA from the CAT vector (pA10CAT2KX) without a DNA insert is initiated at two major and two minor sites. The major band, 210 bases in length, corresponds to the expected initiation site(s) for CAT mRNA. The major band of RNA at 360 bases and two minor bands at 245 and 145 bases show that CAT mRNA also is initiated at aberrant sites. The same initiation sites for CAT mRNA also were found with pA10-N1; however, when the values were normalized to a constant *gpt/trpS/lacZ* mRNA level, the N1 DNA insert was found to increase CAT mRNA synthesis 4- to 6-fold at each of the four mRNA initiation sites detected. Transfection of WOP cells with CAT vector constructs containing W8, W13, and W20 mouse genomic DNA inserts in the 5′ upstream region of pA10CAT2KX resulted in 6-, 5-, and 13-fold increases in CAT mRNA, respectively, initiated from the expected site(s) (≈210 bases) compared to the CAT vector without a DNA insert. A minor band (360 bases) of aberrantly initiated CAT mRNA also was detected in WOP cells. W8 and W20 DNA inserts resulted in 3- and 5-fold increases in the incorrectly initiated CAT mRNA; no increase was detected with W13 DNA.

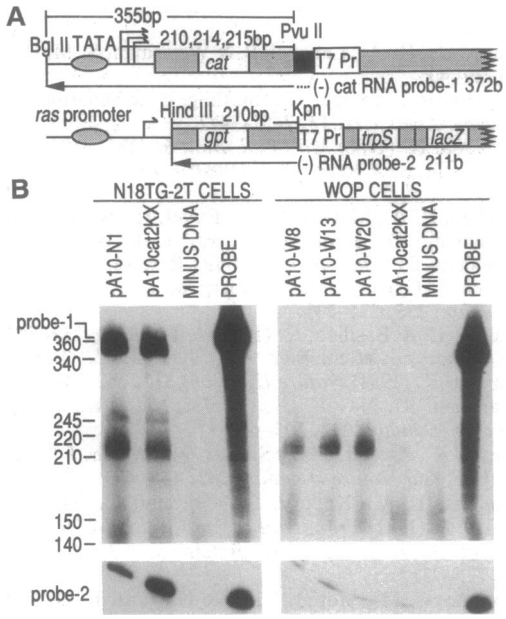

FIG. 3. Effects of mouse genomic DNA inserts subcloned into the enhancerless CAT reporter vector, pA10CAT2KX, on CAT mRNA levels and sites of initiation of CAT mRNA in transfected N18TG-2T or WOP cells. (*A*) Synthesis of (−) CAT mRNA probe 1 and (−) RNA probe 2 transcribed from a chimeric *gpt/trpS/lacZ* gene. T7 represents a T7 RNA polymerase promoter. CAT mRNA synthesis is initiated at three sites when the *cat* gene is linked to the SV40 early promoter. Hybridization of RNA probe 1 to CAT mRNA followed by digestion of single-stranded RNA with RNase T1 was expected to yield three species of RNA, 210, 214, and 215 bases in length. Nucleotide residues 1–17 of probe 1 were transcribed from vector, pSP71 DNA. Probe 2: pRAS-βGAL encodes a constitutively expressed chimeric β-galactosidase gene fused with part of the *E. coli gpt* and *trpS* genes activated by the *ras* promoter. (−) RNA Probe 2 hybridizes to the initial part of *gpt/trpS/lacZ* mRNA. (*B*) N18TG-2T and WOP cells were cotransfected with 10 μg of the recombinant plasmid DNA indicated and 10 μg of pRAS-βGAL DNA or without DNA where indicated. Cells were incubated for 2–3 days; then total RNA was prepared from the cells (25). RNA probes 1 and 2 were hybridized separately with 5 μg of RNA from N18TG-2T cells or 10 μg of RNA from WOP cells; then single-stranded RNA was digested with RNase T1 (26).

Nucleotide Sequences of Clones. Six small cloned mouse genomic DNA inserts were sequenced completely (Fig. 4) and two large clones were sequenced partially (not shown). No strong homology was found between N1, W13, or W20 DNA and sequences in GenBank and EMBL. Nucleotide residues 73–377 of clone W4 exhibit strong homology (90%) to the 3′ long terminal repeat (LTR) of intracisternal A particle (IAP) 14 (29). Three hundred and thirty-two nucleotide residues of clone N106 and 325 residues of the 3′ terminal portion of clone W2 also exhibit strong homology to the 3′ LTR of IAP 14 (94% and 87% similarity, respectively) (data not shown). Therefore, 26.5% of the characterized clones obtained by selection in experiment 2 (Table 1) and 12.5% in experiment 3 are similar to the 3′ LTR of IAP 14. Clone W8 is identical to part of the 5′ flanking region of the mouse vimentin gene (−720 to −598 nucleotide residues) (GenBank accession no. Z22526). In addition, 278 nucleotide residues of the 3′ terminus of clone W5 exhibit 92% homology to the 5′ flanking region of the Syrian hamster cytoplasmic 3-hydroxy-3-methylglutaryl CoA synthase gene (data not shown). Thus, five of the eight mouse genomic DNA clones that were sequenced were identified, and in all cases the homologies found were to regions of DNA that are thought to be involved in the regulation of gene expression.

DISCUSSION

The results show that recombinant plasmids with mouse genomic DNA inserts that activate plasmid DNA replication can be obtained by selection. All DNA inserts examined that increased recombinant plasmid DNA replication also increased expression of a *cat* reporter gene. However, no correlation was found between the extent of the increases in plasmid DNA replication and *cat* gene expression. For example, some DNA inserts markedly stimulated both plasmid DNA replication and *cat* gene expression, whereas other DNA inserts stimulated plasmid DNA replication only slightly but greatly increased expression of the *cat* gene.

Some advantages of the enhancer selection method are as follows. Recombinant plasmid DNA is easily separated from mouse genomic DNA and is readily recovered from transfected mouse cells because plasmid DNA is not integrated in mouse genomic DNA. Deletion of the polyoma enhancer region decreases the rate of replication of pPyEO vector DNA in N18TG-2T neuroblastoma cells or WOP fibroblast cells to relatively low levels (0.8% or 1.7%, respectively, of the values found with pPy-1, which contains an intact polyoma enhancer region). Recombinant clones are selected in two ways: (*i*) enhancers in DNA inserts that increase replication of recombinant plasmid DNA are selectively amplified and (*ii*) plasmid DNA that does not replicate in mouse cells is destroyed selectively by *Dpn* I, while plasmid DNA that has undergone one or more rounds of replication in mouse

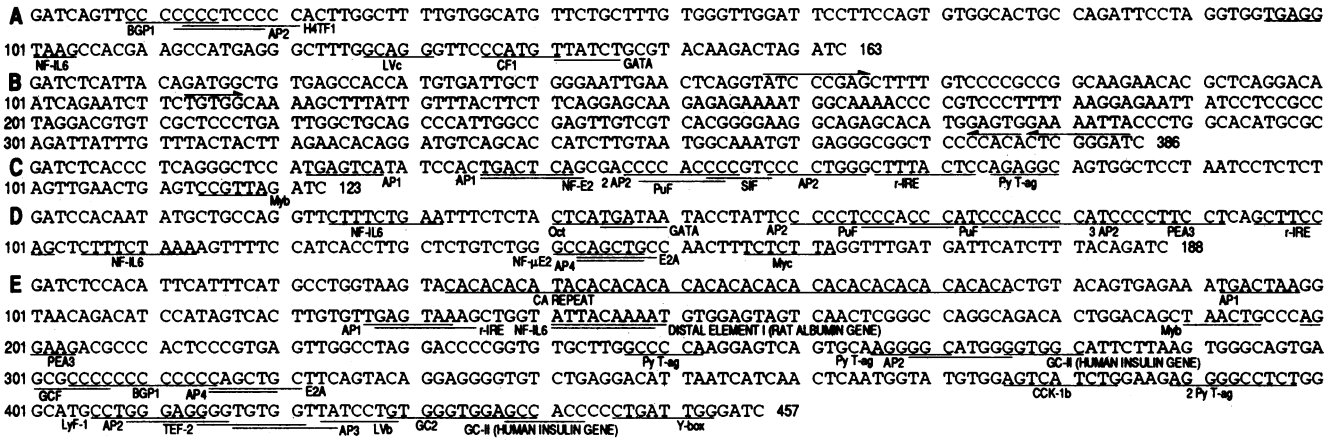

FIG. 4. Nucleotide sequences of mouse genomic DNA clones N1, W4, W8, W13, and W20. The 3′ end of each sequence is adjacent to the polyoma *ori* in pPyEO or the SV40 early promoter in pA10CAT2KX. Putative binding sites for proteins are underlined. (*A*) N1. (*B*) W4. The arrows represent two pairs of inverted repeats. (*C*) W8. (*D*) W13. (*E*) W20.

cells is not cleaved by *Dpn* I. Hence, the enhancer selection method is a sensitive method for selection of recombinant DNA clones.

Possible disadvantages of the enhancer selection method include the following: (*i*) it is not known whether all transcriptional enhancers or only a subset increases plasmid DNA replication and (*ii*) it is likely that the distance between an enhancer sequence in the DNA insert and the polyoma *ori* will affect the activity of the enhancer in stimulating plasmid DNA replication.

We estimate that the abundance of IAP LTR DNA clones obtained by selection in experiments 2 and 3 (Table 1) is at least 540- and 250-fold higher, respectively, than the abundance of the LTR sequences in mouse genomic DNA (30). If we assume that the 5′ upstream region of the mouse vimentin gene is a unique mouse genomic DNA sequence, then two rounds of selection resulted in a >5,000,000-fold increase in abundance of mouse genomic DNA clone W8, which is identical to part of the 5′ upstream region of the mouse vimentin gene. The demonstration that the W8 DNA insert increased the rate of replication of the W8 recombinant 86-fold, which is a greater increase in replication than that found with the polyoma enhancer region, suggests that a highly enriched population of recombinants can be obtained by selection.

It is likely that the enhancer selection method, or a slightly modified method, also can be used to select DNA clones that function as an origin of replication, to select from a library of recombinants with randomly ordered oligonucleotide inserts sequences with high enhancer activity that are targets for DNA binding proteins, and to detect enhancer proteins whose expression may be restricted to certain cell types.

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