Do transcriptional enhancers also augment DNA replication?

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Received July 5, 1988; Revised and Accepted October 26, 1988

ABSTRACT

Enhancers are DNA elements that augment transcription in cis, independent of distance and orientation. Evidence such as hormone dependent neoplastic cell growth and the stimulation of viral replication by sequences present in enhancers suggests that enhancers may also directly affect DNA replication. We tested this hypothesis in recombinant plasmids by asking whether sequences that stimulated DNA replication shared the properties of transcriptional enhancers. The homologous simian virus 40 (SV40) core enhancer was ligated either adjacent to or 2.6 kb distant from the SV40 minimal origin of replication (ori) in both orientations. Plasmids were transfected into T antigen producing COS cells, and episomal DNA was harvested for quantitation of replication. Replication could be assessed either as accumulation of fmol of MboI sensitive progeny DNA, or as a transition in % DNA in replicated (MboI sensitive) versus unreplicated (DpnI sensitive) form. The two measures were related exponentially ($r = 0.86$). The SV40 enhancer augmented replication 1.5-10 fold. The effect was time dependent, distance dependent (only the adjacent enhancer locus stimulated replication), partially orientation dependent, and enhancer copy number independent. Phorbol ester did not affect replication. The heterologous glucocorticoid enhancer had no effect on replication. We conclude that the SV40 enhancer's cis-effect on replication seems to be dependent on the close proximity to the replication origin of specific homologous sequences within the enhancer, rather than a typical enhancer-like effect.

INTRODUCTION

Enhancers are eukaryotic DNA elements that augment transcription in an orientation and distance independent fashion (1,2). Indirect evidence, such as the hormone dependent cell division of some neoplasia (3), suggests that enhancers might also directly affect DNA replication. Indeed, several studies (4-11) have indicated that the presence of papovavirus enhancer elements augments replication from homologous origins of replication.

We tested the hypothesis that enhancers have a cis-effect on replication, using recombinant plasmids containing a simian virus 40 (SV40) minimal origin of replication (ori) as well as either homologous (SV40) or heterologous (glucocorticoid) enhancers, varying orientation, distance, multiplicity, and trans-activation of the enhancers. The SV40 ori functions in mammalian cell nuclei, and may be analogous to the putative replication origins of mammalian chromosomes. Our results suggest that, while the homologous SV40 enhancer does augment SV40 ori-based replication, the effect is clearly differentiated from the classic transcriptional effects of enhancers.

METHODS

Cells

Replication experiments were performed in transfected monkey kidney T antigen producing COS cells (12), while transcription experiments were performed in transfected monkey kidney CV1 cells, in which the SV40 ori does not function unless T antigen is supplied. Cells were grown on 60 or 100 mm plastic dishes at 37°C under 5% carbon dioxide in dialyzed 5% fetal bovine serum, 95% DMEM with 200 units/ml penicillin and 200 ug/ml streptomycin.

Some replication experiments were carried out in a human glucocorticoid receptor producing COS cell derivative, RH5. COS cells were cotransfected with pRSV-hGR (see below) and pTk-neo (13), and then grown in the medium specified above, plus 200 ug/ml G418 (Genetecin, Gibco). G418-resistant foci were cloned. RH5 cells constitutively produce the human glucocorticoid receptor, as shown by dexamethasone-activation of transfected pMtv-luc (see below).

Vectors (see Table 1 and Figure 1)

pSVOACAT (13,14), a hybrid of pBR322, SV40, and the bacterial chloramphenicol acetyltransferase (CAT) gene, was obtained locally.

pORI (from Peter Tegtmeier, Stony Brook, NY), the source of the SV40 minimal ori (8,15), in Table 1, contains the SV40 minimal ori in a pML vector (8,15). Figure 1 details the structure of this SV40 ori.

X-100 (containing the SV40 enhancer, wild type nucleotides 95-272) and X-113 (containing the SV40 enhancer, wild type nucleotides 108-272) were obtained from Paul Berg, Stanford, CA (16). Figure 1 details the structure of these enhancers. Mutants bearing these enhancers, but lacking the SV40 early promoter and ori, are non-transforming and do not produce T antigen (16).

GTCO, from Keith Yamamoto, San Francisco, CA (17), contained a 520 bp mouse mammary tumor virus (mtv) promoterless glucocorticoid response element (glucocorticoid enhancer; GRE) as a BamHI/BglII fragment. GMCO, also from Keith Yamamoto (17), contained a 1450 bp glucocorticoid responsive mtv long terminal repeat (LTR) as a PstI fragment.

pSV2ACAT contains the full SV40 early promoter, enhancer, and ori, and pSV232ACAT contains the full SV40 early promoter and ori, but no enhancer (13,14,16).

pRSV-hGR, expressing the human glucocorticoid receptor cDNA under the control of the strong Rous sarcoma virus (RSV) LTR promoter/enhancer complex, was obtained from Ronald Evans, La Jolla, CA (18).

pMtv-CAT and pMtv-luc, expressing the CAT gene and the firefly luciferase cDNA under control of the glucocorticoid responsive mtv LTR promoter/enhancer complex, were constructed locally by excising the 1450 bp mtv LTR from GMCO (see above) with PstI, followed by blunting of the DNA ends, addition of linkers, restriction digestion, and ligation into either pSVOACAT (14) (to yield pMtv-CAT) or pSVOA-luc (14) (to yield pMtv-luc).

Constructions (see Table 1 and Figure 1)

All recombinant DNA manipulations (restriction digestion, blunting, linker addition, ligation, transformation, etc.) were done by standard procedures (19). Plasmids were grown in the recombination-deficient *E. coli* strain HB101.

1. pORI-CAT and pORIXCAT (SV40 minimal ori plasmids; see Figure 1).

The insert, the SV40 minimal ori from pORI (8,15) was excised as a HindIII/BamHI fragment. The vector, pSVOACAT (13,14) was prepared by BamHI and HindIII digestion of a pSV2ACAT derivative containing only 1 BamHI site. Ligation of insert into vector yielded pORI-CAT. pORIXCAT resulted when the unique HindIII site of pORI-CAT was converted to a unique XhoI site by insertion of an XhoI linker.

2. SV40 minimal ori plasmids bearing the SV40 enhancer adjacent to the minimal ori.

TABLE 1: Plasmid constructions to examine enhancer effects on replication.

Name	Identity	Type	Site in parent	Enhancer		Site in construct	Adjacent or distant
				Number	Orientation to CAT*		
PORI-CAT	Parent plasmid	--	--	--	--	--	--
PORIXCAT	PORI-CAT except HindIII→XhoI	--	--	--	--	--	--
PLXRL	PORI-CAT with 1 proximal SV40 enhancer	SV40	95-272	1	Late	BamHI	Adjacent
PLXLR	"	SV40	95-272	1	Early	BamHI	Adjacent
P2XA	PORI-CAT with 2 proximal SV40 enhancers	SV40	95-272	2	Multiple	BamHI	Adjacent
P2XB	"	SV40	95-272	2	Multiple	BamHI	Adjacent
P3X	PORI-CAT with 3 proximal SV40 enhancers	SV40	95-272	3	Multiple	BamHI	Adjacent
PDE	PORIXCAT with 1 distant SV40 enhancer	SV40	108-272	1	Early	EcoRI	Distant
PDL	"	SV40	108-272	1	Late	EcoRI	Distant
PGR	PORIXCAT with 1 GRE	mtvGRE	-449 → -109	1	Early	BamHI	Adjacent
PGI	"	mtvGRE	-449 -109	1	Late	BamHI	Adjacent

*Early = counterclockwise; late = clockwise (with respect to the counterclockwise CAT transcription unit)

FIGURE 1. Structure of the parent vector, the SV40 min ori, and the enhancers used.

A. The parent vector, pORI-CAT, derived from the pSV series of plasmids (13). The segments are not drawn to proportional scale, so as to highlight functional domains. Text inside the circle denotes the source of the DNA segment. Text outside the circle denotes the function of the DNA segment. The numbers perpendicular to the circumference denote the wild type nucleotide numbers from the sources of the segments (pBR322 or SV40). The triangle in the SV40 ori denotes a deletion, with its boundaries specified in panel B. Early refers to the orientation of the ori segment in wild type SV40. The sites into which enhancers were ligated were the BamHI site adjacent to the ori, or the EcoRI site 2.6 KB distant from the ori. Conversion of the unique HindIII site to a unique XhoI site converted pORI-CAT to pORICAT. The two polyadenylation (A_n) segments upstream of ori prevent readthrough transcription and expression of the CAT from the pBR322 segment of the vector. The chloramphenicol acetyltransferase (CAT) transcription unit (from E.coli Transposon 9), allowing promoter activity of the enhancer/ori constructs to be assayed, terminates in an SV40 small t antigen splice signal and polyadenylation (A_n) signal.

B. The SV40 minimal origin of replication (minⁿori). The SV40 min ori is derived from pOR1 (8,15). It contains only the second T antigen binding site (TagII), and thus lacks T antigen binding sites I and III, found in wild type SV40. The dark triangle represents a deletion of a segment of wild type SV40, with its boundaries noted. Early and late are orientations in wild type SV40.

C. The Enhancers.

i. The SV40 enhancer segment of wild type nucleotide number 95-272 is derived from plasmid X-100 (16). In addition to the two 72 base pair enhancer repeats, it contains a single GC-rich SpI transcription factor binding site (29). Early and late refer to wild type SV40 orientations.

ii. The SV40 enhancer fragment of wild type nucleotides

108-272 is derived from plasmid X-113 (16). The two 72 base pair repeats are shown. Early and late refer to wild type SV40 orientations.

iii. The GRE (glucocorticoid enhancer) is derived from plasmid GTCO (17), and is a 340 bp Sau3A/Sau3A fragment representing an upstream (wild type nucleotides -449 to -109) enhancer from mouse mammary tumor virus (mtv), without promoter activity, containing three binding domains for glucocorticoid ligand-activated receptor.

The vector (pORI-CAT) was linearized at the BamHI site, between the SV40 minimal ori and an A_n site (see Figure 1). The insert, SV40 core enhancer, wild type nucleotides 95-272, was excised from plasmid X-100 (16) as a PvuII/XhoI fragment, blunted, ligated to BamHI linkers, and digested with BamHI to generate BamHI sticky ends. The insert and vector were then ligated at the BamHI site at a molar excess of insert (enhancer) to vector. The multiplicities and orientations of adjacent SV40 enhancers were determined by appropriate digests with restriction enzymes and are shown in Table 1.

3. SV40 minimal ori plasmids bearing the GRE adjacent to the minimal ori. A 340 bp Sau3A fragment of the GRE containing 3 glucocorticoid receptor binding sites from the mtv LTR, but no promoter, was excised from the plasmid GTCO (17), and ligated into the vector pORI-CAT linearized at the BamHI site between the SV40 minimal ori and an A_n site (see Figure 1).

The plasmids generated had single inserts, in either the counterclockwise (5' to 3') (pGR) or clockwise (5' to 3') (pGI) orientation (Table 1).

4. SV40 minimal ori plasmids bearing the SV40 enhancer 2.6 kb distant from the minimal ori.

EcoRI linkers were added to the SV40 enhancer fragment excised from X-113 with PvuII and XhoI (nucleotides 108-272) and the fragment was ligated into an EcoRI site located 2.6 kb away from the minimal ori in pORIXCAT. pDE and pDL represent the early (counterclockwise) and late (clockwise) orientations of the enhancer relative to the minimal ori, respectively.

5. Internal standards.

To correct for equivalent recovery of episomal DNA digested with MboI versus DpnI (see below), an internal standard was included in some gel lanes after digestion. It was generated by cutting the 5497 bp pSV2ACAT with BamHI, yielding fragments of 3522 bp and 1975 bp.

Transfections

Transfected DNAs (10 ug/100 mm plate) (13,14,20) were supercoiled (form I) plasmids sedimented twice on CsCl gradients. Cells were transfected with calcium-phosphate-DNA precipitates as described by Parker and Stark (20).

Some comparative studies used the method of DEAE dextran (21) followed by 100 uM chloroquine (22), wherein a 1 ug total dose of supercoiled DNA was transfected per 60 mm plate.

After some transfections, the effect of glucocorticoid upon replication or transcription was noted by adding 50 nM (final concentration) dexamethasone daily, from a 100 uM stock solution in dimethyl sulfoxide (DMSO).

Because phorbol ester augments the transcriptional effects of the SV40 enhancer (23), the effect of phorbol ester was noted after some transfections by adding 100 ug/ml 12-O-tetradecanoyl phorbol-13-acetate (TPA) at 12 hours, from a 100 ng/ml stock solution in DMSO.

Analysis of Replication

Replication studies were performed in T antigen producing COS cells (12), or a derivative of COS cells that constitutively expresses the human glucocorticoid receptor, the RH5 line (see Cells).

At 24, 48 or 72 hours after transfection, episomal (low molecular weight) DNA was harvested from cell extracts by the method of Hirt (24), and linearized with either HindIII, BamHI, or XhoI. DNA was then cut with either DpnI or MboI. Both enzymes recognize the GATC site, DpnI when the A is methylated by the dam methylase (as it is in plasmid DNA from the dam⁺ E. coli strain HB101, in which plasmids for these experiments are grown), MboI when the A is not methylated (as it is in progeny DNA after two rounds of replication of the plasmid in mammalian cells) (19).

After restriction digestion with MboI or DpnI, the digests were terminated with EDTA and 5 ng of internal standard was added (BamHI digested pSV2ACAT; see Constructions). The purpose of the internal standard was to ensure that equivalent amounts of episomal DNA were digested for calculation of replication by the percent method (see below). The mixture was electrophoresed in 1% agarose in tris-borate-EDTA (19), stained with ethidium bromide (19), photographed, transferred convectively to nitrocellulose sheets (27), and hybridized with a ³²P-labelled, nick-translated (28) probe to visualize linearized versus digested DNA.

Dried blots were autoradiographed on pre-flashed Xomat AR5 film at a series of exposures (1 hour - 10 days) so as to place each band in the linear range for densitometric quantitation (Ultrosan laser densitometer, LKB Produkter, Bromma, Sweden). In addition to the linear bands, the fragment band scanned was an 893 bp MboI or DpnI fragment spanning the CAT gene and the SV40 minimal ori.

Nick-translated (28) probes used to visualize the DNAs were:

1. An 893 bp Sau3A fragment of pORiCAT, spanning the CAT gene and the SV40 ori.
2. The plasmid pORi.
3. Wild type SV40.

Thus, progeny or replicated DNA, after it has gone through at least 2 cycles of replication (so as to have GATC sites on both strands unmethylated, and hence susceptible to MboI cleavage) can in principle be quantitated either as MboI sensitive DNA fragments, or DpnI resistant DNA linears. The relative amounts of progeny (unmethylated) versus parental (methylated) DNA might also be quantitated as % replication, or % of episomal DNA in replicated form:

$$\% \text{ replication} = 100 \left[\frac{\text{replicated plasmid DNA}}{\text{total plasmid DNA}} \right]$$

$$\% \text{ replication} = 100 \left[\frac{\text{MboI sensitive fragments}}{\text{MboI sensitive DNA fragments} + \text{DpnI sensitive DNA fragments}} \right]$$

Analysis of transcription

Transcription studies were performed in monkey kidney CV1 cells, which do not produce the T antigen, so as to remove replication as an independent variable influencing degree of transcription.

48 hours after transfection, cells were lysed and extracted for measurement of soluble protein (2), and either CAT activity (14,26) or luciferase activity (14). Results were normalized to ug protein in the cell extracts.

CAT assays were used to evaluate promoter activity of pORI-CAT and its enhancer-bearing derivatives. Preliminary dose-ranging experiments were performed so that results from each plasmid were detectable (>2 times blank) yet on scale in the assay (<70% of total ¹⁴C-chloramphenicol converted to acetylated derivatives).

Both CAT assays and luciferase assays were used to assess whether the GRE

was activated by glucocorticoid receptor (supplied by pRSV-hGR) plus dexamethasone (50 nM) in the doses used -- for CAT, 1 ug pMtv-CAT plus 9 ug pRSV-hGR, with or without dexamethasone; for luciferase, 1 ug pMtv-luc plus 9 ug pRSV-hGR, with or without dexamethasone. Results are expressed as fold enhancement of transcription by dexamethasone.

Statistics

Results are expressed as the mean value of data from several independent transfections, usually 2-4. Linear, exponential/logarithmic, and polynomial transformations of data were accomplished with a least squares program (Curve Fitter, Interactive Microware, Inc., State College, PA) on an Apple IIc microcomputer. Mean values, standard errors, and t-tests were computed on a Statistics software module of a Texas Instruments TI-99/4A microcomputer. % replication calculations (see section on Replication, above) and internal standard corrections were made by entering the densitometric band data into a spreadsheet (Symphony, Lotus Development Corp., Cambridge, MA) in an IBM PC-AT microcomputer.

RESULTS

Constructions

Figure 1 illustrates the parent plasmid constructed for these studies, pORI-CAT. We also used a derivative of pORI-CAT, called pORiXCAT, in which the unique HindIII site had been converted to a unique XhoI site (see METHODS). The plasmids constructed are listed in Table 1.

Assay of replication

Low molecular weight (episomal) DNA harvested at 48 hours from transfected COS cells could be digested to fragments with either DpnI or MboI (Figure 2). Simultaneous digestion with both enzymes resulted in virtually complete removal of the plasmid linear band (data not shown), suggesting that hybrid intermediates (one parental strand methylated, one progeny strand unmethylated) must be very short lived, or do not accumulate to any detectable degree.

Figure 2 shows the time course of the replication of a recombinant plasmid (p2XB; Table 1) in COS cells. There was a progressive accumulation, with time, of DpnI resistant linear DNA, and MboI sensitive DNA fragments. By contrast, the unreplicated plasmids, before introduction into mammalian cells (Figure 2, left), were completely cut by DpnI but completely resistant to MboI.

In a series of 33 transfections done in one experiment, there was a good correspondence between DpnI resistant linear DNA and MboI sensitive DNA fragments ($r = 0.88$, $n = 33$, $p < 0.001$, $y = 1.19x + 0.584$, where x = fmol of MboI sensitive progeny DNA fragments, and y = fmol of DpnI resistant progeny DNA linears), suggesting that either value could be used to quantitate progeny DNA.

The comparative assessment of DNA replication as % in progeny form (see METHODS) versus mass of accumulated MboI sensitive progeny fragments is shown in an experiment depicted in Figure 3. The correspondence is characterized by an exponential ($r = 0.86$) rather than a linear relationship. The plot could also be fitted to a third order polynomial equation ($r = 0.99$). In any event, in this experiment it is clear that the % method does not discriminate different degrees of accumulation of replicated mass of progeny DNA after the % figure passes 60-70%. A similar exponential relationship ($r = 0.78$, $n = 39$, $p < 0.01$, $\log_{10} y = 0.0391x - 2.48$, where x = replication as % in progeny form, while y = replication as mass of progeny DNA in fmol) was noted in another experiment involving 39 transfections.

Figure 3 and Table 2 indicate that pSV2ACAT, containing the full wild type SV40 ori, replicates to a much greater extent than the minimal ori plasmids

FIGURE 2. Time-dependent accumulation of DpnI resistant, MboI sensitive progeny DNA after transfection of p2XB (10 ug, calcium phosphate method) into COS cells. Episomal DNA was harvested at 24, 48 and 72 hours, linearized with BamHI, cut with MboI or DpnI, adjusted with internal standard (BamHI cut pSV2ACAT, yielding fragments of 3522 bp and 1975 bp detected by the probe), electrophoresed in 1% agarose, transferred to nitrocellulose, probed with a 32 P-labelled, nick translated 893 bp Sau3A fragment of pORIXCAT (spanning the CAT gene and the SV40 ori), and autoradiographed. The input plasmid (left side) is resistant to MboI but fully cut by DpnI. Progeny DNA (right side) accumulates in time dependent fashion, as DpnI resistant linears, or MboI sensitive fragments.

pORI and pORIXCAT, and that qualitatively similar results are obtained in calcium phosphate or DEAE dextran transfections of plasmid DNAs.

Effect of adjacent SV40 enhancers on replication

The replication effect of SV40 enhancers adjacent to the SV40 minimal ori (in the BamHI site, Figure 1) is shown in Figure 4. One, two or three copies of the core enhancer each augmented replication, and the augmentation was 1.5-10 fold of the value for pORIXCAT (Figure 4). The enhancer-dependent augmentation of progeny DNA was progressive with time (Figure 4, left). The enhancement of replication occurred in both orientations, although the effect

FIGURE 3. Correspondence of two methods for determining extent of DNA replication. The results are from 11 separate transfections, in one experiment, of 1 ug of DNA by the DEAE-dextran method. The twelfth point is the reference origin (0,0) for unreplicated DNA. Episomal DNA was harvested 48 hours after transfection. Southern blots, as in Figure 2, were densitometrically scanned. % plasmid DNA in replicated form

$$(100) \left[\frac{\text{MboI sensitive progeny DNA fragments}}{\text{MboI sensitive progeny DNA fragments} + \text{DpnI sensitive parental DNA fragments}} \right]$$

was plotted versus accumulated mass of MboI sensitive progeny DNA fragments, in fmol. The data could be fitted to either an exponential or third order polynomial equation.

was somewhat greater for the early (p1XLR) enhancer orientation, especially at 48 and 72 hours. This orientation reconstructs the normal, wild type orientation of the enhancer to the ori. The enhancement of replication was not dose-dependent upon enhancer copy number (1, 2 or 3 concatenated). When assaying replication as % of DNA in progeny form (Figure 4, right), the stimulation of replication was maximal at 24 hours, and virtually disappeared by 72 hours. This apparent loss of the enhancer effect simply illustrates the hazards of the % replication method at % values of >60-70% -- the vertical portion of the exponential curve (Figure 3) indicates that the % value no longer linearly mirrors accumulation of progeny DNA.

Effect of distant SV40 enhancers on replication

When the SV40 enhancer was inserted into an EcoRI site 2.6 kb distant from the minimal ori (Figure 1), there was no enhancer effect on replication (Table 3, Figure 5) in either enhancer orientation (early vs. late). Indeed, at 72 hours there was an apparent suppression of basal replication by up to one-half (Table 3).

Effect of TPA on replication and the SV40 enhancer's effect

A matrix of 16 transfections was done to evaluate systematically the effects of the adjacent SV40 enhancer (late, p1XRL orientation), % serum (0.5

TABLE 2: Replication of test plasmids: stratified by method of transfection*

Plasmid	Ca ₃ (PO ₄) ₂ transfection(10 ug)	DEAE-dextran transfection(1ug)
	Progeny DNA ₁₆ moles x 10 ⁻¹⁶	Progeny DNA ₁₆ moles x 10 ⁻¹⁶
pOR1	4.5	1.4
pOR1xCAT	1.8	1.3
pSV2ACAT	17.8	15.3

*Mean values of duplicate experiments, with episomal DNA harvested at 48 hours

vs. 5%), transfection DNA dose (1 versus 10 ug) and TPA (100 ng/ml) on replication at 24 hours post transfection (Table 4).

TPA and varying % serum had no effect on degree of replication. The SV40 enhancer (p1XRL) did augment replication as noted previously. A 10 ug transfection dose yielded more fmoI of replicated progeny DNA than the 1 ug

FIGURE 4. Effect of adjacent (BamHI site) SV40 enhancer(s) on DNA replication in recombinant plasmids. The parent plasmid, without enhancer, is pOR1xCAT. Left, replication as assessed by accumulated mass of progeny DNA (MboI sensitive progeny fragments). Right, replication as assessed by % of episomal DNA in progeny form [(100)(MboI sensitive progeny fragments/MboI sensitive progeny fragments + DpnI sensitive parental fragments)]. The results are the mean values from 3 separate transfection experiments.

Plasmid	SV40	Enhancer	Progeny DNA			Replication, as %		
	enhancer	orientation (to CAT)	moles x 10 ⁻¹⁶ 24h	48h	72h	in progeny form 24h	48h	72h
pORixCAT	-	--	5.9	11.5	11.8	31.6	67.8	83.3
pDE	+	Early	5.0	8.9	6.7	21.1	55.7	48.2
pDL	+	Late	8.7	9.5	5.4	15.4	49.5	50.4

plasmid dose (5.8 ± 0.6 versus 4.2 ± 0.6 moles $\times 10^{-16}$, $p = 0.015$), but the response at 10 μg was clearly nonlinear (that is, not 10 times the response at 1 μg).

In COS cells, the GRE had no effect on replication in either enhancer orientation, either in the basal state or after activation with glucocorticoid receptor plus glucocorticoid (Table 5). Glucocorticoid alone had no effect on replication.

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TABLE 4: Effect of phorbol ester (TPA), the SV40 enhancer, % serum and transfection DNA dose on replication*

Comparison	Category	Progeny DNA ₁₆ moles x 10 ⁻¹⁶ (mean +/- SEM, (n=8 ea))	p value**
Dose of plasmid	1 ug	4.2 + 0.6	0.015
DNA transfected	10 ug	5.8 + 0.6	
% serum in medium	0.5%	5.3 + 0.6	Not sig- nificant
	5%	4.6 + 0.7	
Absence/presence of SV40 enhancer	pORiXCAT	4.0 + 0.4	0.015
	pIXRL	5.9 + 0.7	
TPA, presence/ absence at 100 ng/ml	-	4.8 + 0.7	Not sig- nificant
	+	5.2 + 0.6	

* Cells extracted 24h after transfection

** paired t-test

evaluated transcriptional activation of the GRE in CV1 cells by cotransfection of pRSV-hGR (9 ug) with either pMtv-CAT (1 ug) or pMtv-luc (1 ug). The mtv plasmids encode the mtv LTR, including GRE and promoter, upstream from the reporter genes CAT or luciferase. Exposure of the cotransfected (pMtv-CAT plus pRSV-hGR) cells to dexamethasone (50 nM) augmented transcription 2290 fold from the CAT reporter gene (mean value from two separate sets of transfections). Similar dexamethasone induction results were also noted for the luciferase reporter (data not shown). Thus, our experimental conditions were sufficient to activate the glucocorticoid enhancer's transcriptional effects. Dexamethasone alone, without enhancer or receptor, did not influence CAT or luciferase activity.

RH5 cells are a derivative line of COS which also constitutively expresses the human glucocorticoid receptor (see METHODS), and in which transcription of pMtv-luc is similarly augmented by dexamethasone. The GRE did not influence replication in these cells, either (Table 5). An apparent difference in degree of replication of all plasmids in COS versus RH5 cells (Table 5) is of uncertain significance, since the two different cell types may not have been transfected at exactly the same position on their cell growth curves.

Transcriptional effects of the enhancers

The CAT reporter transcription unit in our constructs (Figure 1) afforded the opportunity to see whether the enhancers we ligated into the product plasmids (Table 1) also had promoter activity. The results were analyzed not only as enzymatic activity (% of ¹⁴C-chloramphenicol acetylated per unit time, normalized to ug cell protein), but also as fold augmentation of transcription versus the parent, enhancerless plasmid pORiXCAT, and as % of transcription programmed by pSV2ACAT, a plasmid with a full wild type SV40 enhancer and early promoter. pORiXCAT programmed CAT activity 0.025 % as well as pSV2ACAT (mean result from 2 separate sets of transfections).

The adjacent SV40 enhancer constructs all displayed more transcription than the parent, enhancerless pORiXCAT, though far less than (i.e., 1.6-3.0 % of) the amount of transcription programmed by pSV2ACAT (data not shown). This is the expected result since the minimal ori retains only a crippled promoter,

TABLE 5: Effect of adjacent (BamHI site) glucocorticoid enhancer (GRE) on replication* of test plasmids

Cell line	Test plasmid		Glucocorticoid enhancer** (to CAT)	Enhancer orientation (to CAT)	Other plasmid cotransfected		Non-specific carrier DNA ug transfected	Dexamethasone, 5x10 ⁻⁸ M	Progeny DNA as % in progeny form			Replication, as % in progeny form		
	Name	ug			Name	ug			mole x 10 ⁻⁶			mole x 10 ⁻⁶		
									24h	48h	72h	24h	48h	72h
COS	pORiXCAT	10	--	--	--	--	--	--	3.0	8.7	10.0	21.8	53.4	80.8
COS	"	10	--	--	--	--	--	+	5.7	13.5	6.7	25.3	61.8	54.8
COS	"	1	--	--	--	--	9	--	2.7	---	---	40.5	---	---
COS	pGR	1	+	Early	pRSV-hGR	9	--	--	1.6	0.9	1.9	23.3	36.3	60.1
COS	"	1	+	Early	"	9	--	+	1.0	1.1	1.9	34.3	40.4	54.7
COS	pGI	1	+	Late	pRSV-hGR	9	--	--	2.4	4.7	3.1	33.7	55.0	65.0
COS	"	1	+	Late	"	9	--	+	1.2	1.8	3.2	15.2	46.3	67.2
RH5**	pORiXCAT	10	--	--	--	--	--	--	27	100	95	58.9	81.5	89.5
RH5	pGR	1	+	Early	--	--	9	--	87	131	83	68.7	85.1	79.4
RH5	"	1	+	Early	--	--	9	+	42	84	64	40.4	71.5	82.3
RH5	pGI	1	+	Late	--	--	9	--	92	138	131	56.4	76.3	93.4
RH5	"	1	+	Late	--	--	9	+	105	157	205	72.4	76.7	82.0

* Mean values of two separate experiments whose total DNA transfected was 10 ug

** RH5 = COS cells constitutively expressing pRSV-hGR from integrated copies of the human glucocorticoid receptor gene

*** The glucocorticoid enhancer (GRE) is mtv wild type nucleotides -449 to -109

lacking SpI binding sites. For the adjacent SV40 enhancers, the augmentation of CAT transcription (versus pORiXCAT) did not correlate significantly with the augmentation of replication at the same 48 hour time point ($r = 0.34$, $n = 5$, $p > 0.1$).

The distant SV40 enhancers (in pDE and pDL) had little effect on CAT transcription (0.100-0.103 % of pSV2ACAT). Similarly, the adjacent glucocorticoid enhancers (in pGI and pGR), with or without glucocorticoid plus receptor, did not augment CAT activity (0.0006-0.005% of pSV2ACAT).

DISCUSSION

Several previous studies have indicated that the SV40 enhancer may augment SV40 ori-based DNA replication (4-11). We undertook the present experiments to determine whether this function of the enhancer was analogous to its transcriptional effects (1,2).

Our results (Table 3; Figures 4 and 5) indicate that the SV40 enhancer in cis does augment DNA replication, in a time dependent, copy number independent, distance dependent, and partially orientation dependent fashion. Our orientation and distance effects are consistent with those of a previous report (11).

Several features differentiate this effect from the classic effects of transcriptional enhancers (1,2):

1. The effect is copy number independent (Figure 4).
2. The effect is distance dependent, virtually disappearing when the SV40 enhancer is moved 2.6 kb from the ori (Table 3, Figure 5).
3. The effect is not augmented by phorbol ester (Table 4), an agent that trans-activates the SV40 enhancer's effect on transcription (23).
4. The effect is not reproduced by a heterologous enhancer, the GRE (Table 5), even when appropriately trans-activated.

It is unlikely that the adjacent SV40 enhancer exercised its effects on replication within the plasmid simply as a "spacer", separating the SV40 ori from prokaryotic pBR322 sequences (Figure 1), since the 340 bp GRE in the same adjacent BamHI site did not affect replication. Prokaryotic pBR322 sequences which "poison" SV40 replication (34) are unlikely to have played a major role in the results presented here, since pOR1 (which lacks the "poison" sequences (8)) and pORiXCAT (which contains the "poison" sequences (Figure 1)), replicated to similar extents (Table 2; Figure 3). However, we did not directly or specifically examine the effect of "poison" sequences in these experiments.

The two methods of estimation of DNA replication -- % in replicated (progeny) form versus accumulated mass of progeny DNA (Figure 3) -- each have advantages. The % method gives a dimensionless value that is not affected by vagaries of episomal DNA recovery from transfected cells. The mass method gives more accurate information about substantial replication beyond 60-70% of the total, as would occur after multiple rounds of replication.

Were the replication effects of the SV40 enhancer caused by increased transcription moving through the ori? The adjacent (BamHI site; Figure 1) SV40 enhancer constructs did augment CAT transcription as well as SV40 ori-based replication (Figure 4). Indeed, the adjacent SV40 enhancer (SV40 wild type nucleotides 95-272) does contain a single GC rich binding site for the transcription factor SpI (29), a component of the SV40 early promoter, as indicated by the box at the "early" end of the enhancer diagram in Figure 1. In addition, the SV40 late promoter is virtually inseparable from sequences within the SV40 enhancer (30). Finally, short RNA primers have been implicated as transient intermediates in initiation of DNA replication (31). However, several observations argue against a replication effect simply secondary to increased transcription:

1. Augmentation of transcription did not correlate significantly with

augmentation of replication in these experiments ($r = 0.34$, $p > 0.1$).

2. SV40 ori-based DNA replication is not affected by alpha-amanitin, an inhibitor of RNA polymerase (32, 33).

3. An adjacent heterologous promoter does not affect SV40 ori-based DNA replication (8).

4. pSV2ACAT and pSV232ACAT, which exhibit a 50-100 fold difference in transcription, replicate to similar extents (data not shown).

A previous report (5) suggesting that a heterologous enhancer could activate polyoma virus replication in cis could well have been a trans-effect through augmented T antigen production.

The nature of the SV40 enhancer effect on the SV40 ori thus remains obscure. Future experiments might clarify whether transcription factors binding to the SV40 enhancer (35) or replication factors binding outside the ori (example, in the early promoter/early enhancer region (32)) sensitize the ori, perhaps by alteration of SV40's chromatin or nucleosome structure at the ori (36). In fact, protein factors NF1 and NF3, known to be involved in adenovirus DNA replication, are also known to bind to transcriptional enhancers (37,38). However, further work is required to establish whether these or other proteins are involved in the enhancement of DNA replication by sequences residing in the SV40 enhancer (39).

ACKNOWLEDGEMENTS

We appreciate the help of those in our laboratory who began or completed many of the plasmid constructions used in these studies -- Patricia Menzel, Karen Deyerle, J. Aaron Cassill, and Stephen Gould. Supported by the Veterans Administration, the National Institutes of Health, and the American Heart Association. Dr. O'Connor was an Established Investigator of the American Heart Association. Dr. Subramani is an RCDA of the National Institutes of Health.

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