DNA replication facilitates the action of transcriptional enhancers in transient expression assays

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ABSTRACT

We demonstrate a general role for DNA replication in the activation of gene transcription in transient transfection assays. The effect is observed for a wide range of genes and cell types, transfected by a number of protocols and is independent of increased template copy number. Replication does not stimulate transcription driven by proximal promoter elements alone but requires a functional enhancer element. This synergy between an active replication origin and an enhancer is not confined to elements from viruses such as SV40, which undergo an early to late switch in gene expression that is tightly coupled to replication, since the enhancer-containing long terminal repeats from retroviruses are strongly stimulated by replication. Furthermore, synthetic enhancers consisting of multimerised binding sites for one or two factors are also subject to replication-activation. The diversity of synthetic and natural enhancers used in this study suggests that replication and transcription do not share a common protein factor. We propose that replication leads to chromatin modifications that facilitate enhancer action.

INTRODUCTION

DNA replication has been implicated in both the alteration and maintenance of gene expression programmes during differentiation and development (1–6). The competitive relationship between chromatin assembly and the formation of active transcription initiation complexes is becoming increasingly well documented (reviewed in 7), and since both structures could be disrupted by replication, an opportunity for reprogramming of the genome is thereby created (3,8,9). Furthermore, the correlation between the transcriptional activity of many different genes and the timing of their replication within S phase suggests that any reprogramming function of replication may be controlled in a temporal manner (1).

Evidence in favour of a stimulatory role for replication was obtained in a transient transfection assay using the *Xenopus* β globin gene linked to SV40 sequences (10). We demonstrated

that the effect was not due simply to changes in template copy number or covalent modification, but rather reflected a mechanistic contribution to transcriptional activation. Here we show that activation of transcription by replication is a general phenomenon for genes linked to an enhancer. Enhancer-independent activation cues, such as SV40 T antigen transactivation, function independently of DNA replication. This synergy between enhancement and replication is common to a number of enhancers including synthetic sequences constructed from multimerised binding sites of a single enhancer factor. These data argue that replication contributes to the establishment of a productive interaction between an enhancer and its target promoter.

MATERIALS AND METHODS

Plasmid constructions

pSVEO⁺ and *pSVEO⁻⁽⁶⁻¹⁾*: The 342bp HindIII to PvuII fragment spanning the SV40 control region (coordinates 5171–272), from pSV β 183' and pSV β 183'orim (10), was inserted into the HindIII and AccI (end-filled with Klenow polymerase) sites of pUC18.

pB/STSVEO⁺ and *pB/STSVEO⁻⁽⁶⁻¹⁾*: The 366 bp HindIII to KpnI fragment spanning the SV40 control region (coordinates 5171–294) was taken from pSV β 183' and pSV β 183'orim and inserted into the same sites of pBluescript KS⁺ (Stratagene).

pUCori⁺ and *pUCori⁻⁽⁶⁻¹⁾*: The 200bp HindIII to SphI fragment containing the SV40 origin and early promoter (coordinates 5171–128), from pSVEO⁺ and pSVEO⁻⁽⁶⁻¹⁾ was inserted into the same sites of pUC18. pX β CAT was constructed by inclusion of the CAT gene and SV40 small t intron and polyadenylation site from pSV2CAT (11), into the promoter subclone pUC β 521 (constructed by Dr Alex Argirolakitis). pUC β 521 contains the *Xenopus* β globin promoter from the EcoRI site (–478) to the (end-filled) NcoI site (+50) inserted into the EcoRI and SmaI sites of the pUC18 polylinker, and this fragment includes the globin initiation codon (+47) which is within the NcoI site. The NcoI site is regenerated in this fusion. This subclone was linearized with BalI which cuts just upstream of the NcoI site in the promoter (+44), and recut with AatII in the plasmid. The

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CAT cassette, as a 2455bp HindIII (Klenow end-filled) to AatII fragment, was inserted to generate pX β CAT. The first initiation codon following the transcription start site is that of the CAT gene.

pX β CATEO⁺ and pX β CATEO⁻⁽⁶⁻¹⁾ were constructed by replacing the BamHI-AatII fragment downstream of the CAT gene in pX β CAT with the 820 bp BamHI-AatII fragments containing the SV40 enhancer/origin cassettes from pSVEO⁺ and pSVEO⁻⁽⁶⁻¹⁾ respectively.

pX β CATori⁺ and pX β CATori⁻⁽⁶⁻¹⁾ were constructed from pX β CAT by replacing the downstream BamHI-AatII fragment with 700 bp BamHI-AatII fragments containing the wild type and mutant SV40 origin cassettes from pUCori⁺ and pUCori⁻⁽⁶⁻¹⁾ respectively.

pX β CAT6xA/A⁺, pX β CAT6xA/C⁺, pX β CAT6xB20⁻, pX β CAT6xdpm2⁻ and pX β CAT6xC16⁺ were constructed by replacing a 200 bp HindIII-PstI fragment containing the mutant SV40 origin from pX β CATori⁻⁽⁶⁻¹⁾ with 120 bp HindIII-PstI fragments containing six copies of various natural and artificial enhancer pairings derived from the SV40 enhancer. These were taken from p β 6xcoreA/coreA⁺, p β 6xcoreA/coreC⁺, p β 6xB20wt⁻, p β 6xB20dpm2⁻ and p β 6xC16⁺ respectively in which the multimers are cloned into the SphI site downstream of the human β globin gene in p β e⁻ (Gifts of Drs Winship Herr and Masafumi Tanaka, Cold Spring Harbor Laboratory, described in 12,13).

pX β CAT6xA/A⁺ori⁺, pX β CAT6xA/C⁺ori⁺, pX β CAT6xB20⁻ori⁺, pX β CAT6xdpm2⁻ori⁺ and pX β CAT6xC16⁺ori⁺ contain the SV40 origin of replication cassette downstream of the enhancer multimers as a HindIII (end-filled with Klenow)-AatII fragment from pUCori⁺ inserted into the HindIII (end filled)-AatII sites of each of the pX β CAT6xmer plasmids. The equivalent series containing the mutant (6-1) origin were produced in the same way using pUCori⁻⁽⁶⁻¹⁾. The structures of the synthetic enhancer repeats which are separated by XhoI recognition sites are shown in Fig. 6.

ph β CAT (a gift of Dr Dave Greaves, National Institute of Medical Research) was made by replacing the AccI-HindIII fragment containing the SV40 control region from pSV2CAT with an 830 bp HpaI-HindIII fragment containing the human adult β globin promoter from construct man6H. The HpaI site is at -800 relative to the transcription start site and the HindIII site is at +30. ph β CATEO⁺ and ph β CATEO⁻⁽⁶⁻¹⁾: BamHI-AatII fragments from pSVEO⁺ and pSVEO⁻⁽⁶⁻¹⁾ containing the enhancer/origin cassettes were inserted into the same sites downstream of the CAT gene of ph β CAT.

pB/STSVEO⁺CAT and pB/STSVEO⁻⁽⁶⁻¹⁾CAT resemble pSV2CAT in containing the CAT gene fused to the early (HindIII) side of the SV40 control region. They were constructed by insertion of a HindIII-PstI fragment from pSV2CAT containing the CAT gene and SV40 splice and polyadenylation signals into the same sites in the polylinker of pB/STSVEO⁺ and pB/STSVEO⁻⁽⁶⁻¹⁾ respectively.

pRSVCAT derivatives: The wild type and mutant SV40 origin cassettes from pUCori⁺ and pUCori⁻ were inserted as BamHI-AatII fragments into the same sites downstream of the CAT/SV40 gene in pRSVCAT (14) to generate pRSVCATori⁺ and pRSVCATori⁻ respectively.

pMSVCAT and derivatives: pMSVCAT is described in Gorman et al. (15). It contains a 569 bp HinfI-SmaI fragment containing the complete 3' Murine Sarcoma Virus LTR placed upstream of the CAT gene. The wild type and mutant SV40 origin cassettes

from pUCori⁺ and pUCori⁻ were inserted as BamHI-AatII fragments into the same sites downstream of the CAT/SV40 gene in pMSVCAT to generate pMSVCATori⁺ and pMSVCATori⁻ respectively.

pSV δ RB (constructed by Dr Tariq Enver) is a 4.5 kbp EcoRI-BamHI fragment containing the entire SV40 control region and early transcription unit in the pBR322 derivative pJYM.

pSVXL3 and pXSTOP (gifts of Dr Nic Jones, Imperial Cancer Research Fund): pSVXL3 is an XhoI linker mutant of the Adenovirus 5 E1A gene. The insertion of an 8 nucleotide-long XhoI linker in the amino terminal end of the 289 amino acid protein ORF shifts the coding sequence reading frame resulting in a truncated peptide of 70 amino acids. The mutated E1A gene including all upstream sequences was inserted as an EcoRI-PstI fragment into pSVod which contains the SV40 minimal origin (16). pXSTOP is a derivative of pJOAC which is the left-hand 7.8% of the Adenovirus 5 genome inserted into the HindIII site of pBR327. Base 933 of the viral insert was mutated from A to T creating a unique XhoI site. pXSTOP was produced by deleting the internal XhoI (933) to XbaI (1340) fragment. This generates a frameshift at amino acid 127 and immediate truncation (17).

Cell culture and transfection

Human HeLa, Daudi, K562 and mouse L cells were maintained in RPMI 1640 medium with Hepes (GIBCO), supplemented with penicillin (100U/ml), streptomycin (100 μ g/ml), 2mM L-glutamine and 10% fetal calf serum in air at 37°C. DEAE-dextran transfection was performed essentially as described in Gorman (18) and included a 15–17% glycerol shock step to increase uptake and expression (19).

Electroporation was performed on trypsinized HeLa cells using a Hoeffer ProGenetor (PG101) electroporation pulse controller, 2.5 cm high single-ring electrode and PS500X DC power supply. Cells were washed in ice-cold phosphate buffered saline (PBS) and resuspended in phosphate buffered sucrose (PBSu) shortly before transfection. PBSu is 272 mM sucrose, 7 mM sodium dihydrogen phosphate, 1 mM magnesium chloride (pH 7.4) and was sterilized by autoclaving (20). Approximately 4 $\times$ 10⁶ cells were used per individual transfection and suspended in a total volume of 1 ml of PBSu loaded with 2–5 μ g of specific DNA along with 500 μ g sheared salmon sperm carrier DNA. Cells were voltage shocked using a time constant tau of 7 msec and a voltage of 408V giving a field strength (E) of $\sim$ 740 Vcm⁻¹.

For the modified scrape loading, or 'scrapefection' method, the growth medium was removed and the cells were washed twice in PBS. The DNA mixes were made up to 1.2 mls in tris buffered saline (TBS, pH 7.4), filter sterilized and layered onto the cells. After a 10 min incubation on ice, the cells were scraped off the plate surface, suspended in 5mls of RPMI with 10% fetal calf serum (at 4°C), transferred to a conical Sterilin tube and pelleted by centrifugation. The cell pellets were resuspended in 10 mls of fresh RPMI medium with 10% fetal calf serum and replated for 2 days.

Recovery and analysis of RNA and DNA from transfected cells

Cells were recovered and washed as described (19). RNA was prepared by the 'single-step' method of Chomzynski and Sacchi (21) and analysed by primer extension (22). The Adenovirus type 5 E1A oligonucleotide primer was 5' CTGGCGGCCATTCTT-CGG 3' which is complementary to sequences from +94 to +113 (a gift of Dr Nic Jones, Imperial Cancer Research Fund). Total

RNA from half a confluent 9 cm plate (about 2.5×10^5 cells) was used per assay. Annealing was at 52°C and reverse transcription was at 42°C . Samples were ethanol precipitated, fractionated on a 6% polyacrylamide sequencing gel and visualised by autoradiography.

Transfected DNA was recovered by Hirt extraction, digested with an enzyme that linearised the plasmid and analysed by Southern blotting (19). Relative levels of plasmid DNA were determined by scanning densitometry of suitable autoradiographs using a Molecular Dynamics 330A Computing Densitometer. To establish the fraction of recovered DNA that had been produced by replication, samples were further digested with the methylation-dependent restriction enzyme Dpn I. Templates that have undergone one or more rounds of replication in eukaryotic cells become resistant to digestion with Dpn I (10). Replication efficiency varied but averaged 30% of total recovered template population. The overall increase in copy number did not exceed 2-fold in any of the human cells used in this study.

Analysis of CAT activity

Cell extracts were prepared and assayed for CAT activity as described by Gorman (18) except that lysates were incubated at 60°C for 5 min to eliminate endogenous deacetylase activities (23). Percentage acetylation was determined either by cutting the spots from the plate and counting in a scintillation counter, or by scanning densitometry.

RESULTS

DNA replication promotes β globin gene activation in a number of different cell types

We showed previously that replication activates transcription of the *Xenopus* β globin gene linked to the SV40 enhancer in transfected HeLa cells, irrespective of changes in template copy number or modification (10). To investigate the generality of the effect, we examined the importance of replication in a number of other cell lines which support replication from an SV40 origin. In addition, to determine if this was a peculiarity of the *Xenopus* gene, we examined the effect of replication on human β globin gene expression.

To facilitate analysis, the *Xenopus* and human β globin gene promoters were fused to the bacterial chloramphenicol acetyl transferase (CAT) gene (constructs pX β CAT and ph β CAT; see Fig 6A). Cassettes containing both the SV40 enhancer, which is required for β globin expression in non-erythroid cells (10, 24), and either a wild type or an inactive mutant (6-1) SV40 origin of replication (25) were inserted downstream of the transcription units (constructs pX β CATEO⁺, ph β CATEO⁺, pX β CATEO⁻⁽⁶⁻¹⁾ and ph β CATEO⁻⁽⁶⁻¹⁾; see Fig 6A). T-antigen, which is absolutely required for replication from the SV40 origin, was supplied by co-transfection of a plasmid containing the SV40 early region (pSV δ RB), and replication of transfected DNA was monitored using a methylation-sensitive restriction enzyme (see Materials and Methods).

When these constructs were transiently transfected into HeLa cells using the DEAE-dextran protocol, the dramatic combined effect of the enhancer and replication, reported previously using the intact *Xenopus* β globin gene (10), was clearly apparent for both the *Xenopus* and the human promoters (Fig 1, constructs β EO⁺ + T-antigen). Ribonuclease protection analysis confirmed that the increased CAT activity of replicating enhancer constructs reflected an increase in correctly initiated RNA

Figure 1. Synergistic activation of β globin promoters by the SV40 enhancer and origin of replication as analyzed by CAT activity. Representative CAT assays are shown for extracts prepared from HeLa cells transfected with 0.5 pmoles ($\sim 1.75 \mu\text{g}$) ph β CAT, h β CATEO⁻⁽⁶⁻¹⁾, ph β CATEO⁺, pX β CAT, pX β CATEO⁻⁽⁶⁻¹⁾ and pX β CATEO⁺. Constructs are diagrammed in Fig 6A. Each reporter was cotransfected with 0.25 pmoles of either pSV δ RB which supplies SV40 T/t antigen (+ sign) or pSVEO⁺ which does not (- sign). The levels of CAT activity measured as percentage acetylation are displayed below in the form of a histogram.

transcripts (data not shown). This experiment demonstrates that the replication effect is not confined to the *Xenopus* β globin gene and that only the promoters of the genes are required for the response. An internal reference plasmid was not included in these experiments because of interference through T-antigen transactivation (see below) and through promoter competition, especially for the weak signals in the absence of replication. Furthermore, the low levels of replication in these semi-permissive cells meant that a replicating internal control also interfered through competition for the replication apparatus. Therefore, to control for experimental variation, all transfections were performed independently between three and five times, and template numbers were carefully monitored. Quantitative data in the figures refer to the representative examples shown.

Figure 2. Synergistic activation by the SV40 enhancer and DNA replication in human B-cell and erythroid cell lines. SV40 T/t antigen was supplied by 0.25 pmoles of pSVδRB where indicated (+). (A) Human Daudi cells (pre-B lymphoma cell line) transfected with pXβCAT (lanes 1,2), pXβCATEO⁻₍₆₋₁₎ (lanes 3,4) and pXβCATEO⁺ (lanes 5,6). (B) Human K562 cells (embryonic/fetal erythroleukaemia cell line) transfected with 0.5 pmoles of pXβCAT (lanes 1,2), pXβCATEO⁻₍₆₋₁₎ (lanes 3,4), pXβCATEO⁺ (lanes 5,6), phβCAT (lanes 7,8), phβCATEO⁻₍₆₋₁₎ (lanes 9,10) and phβCATEO⁺ (lanes 11,12). (C) Murine L-cells (fibroblast-like) were transfected with 0.5 pmoles of pXβCAT (lanes 1,2), pXβCATEO⁻₍₆₋₁₎ (lanes 3,4) and pXβCATEO⁺ (lanes 5,6).

Results obtained in CV-1 cells and also in their T antigen-producing derivatives Cos-7 (26) were similar to those found in HeLa cells (not shown). Figure 2 extends the observations to two human non-fibroblastic cell lines. In the pre-B cell line, Daudi, replication stimulated expression from the enhancer-containing *Xenopus* β globin construct by 8 to 10-fold (Fig 2A, compare lanes 4 and 6). In the erythroid cell line, K562 (panel B), the SV40 enhancer stimulated the *Xenopus* β globin promoter ~25-fold without replication (compare lanes 1,2 with lanes 3,4), nevertheless replication further elevated this activity by >50-fold (lane 6). Thus, despite an initially high level of enhancer activity, DNA replication still substantially increased the level of stimulation. In contrast to the *Xenopus* promoter, expression from the human β globin promoter was low in K562 cells. It has been well documented that both the endogenous and transfected adult human β globin genes are negatively regulated in these embryonic/foetal erythroid cells, presumably reflecting

Table 1. Synergism between the SV40 enhancer and DNA replication following transfection of HeLa cells by electroporation (E) or scrapefection (S).

Method	Construct	Amount transfected (pmoles)	CAT activity (% acetylation)
E	pXβCATEO ⁻ ₍₆₋₁₎	1.0	0.9
E	pXβCATEO ⁺	1.0	33.7
S	pXβCAT	0.5	0.26
		1.0	0.43
		2.0	0.25
S	pXβCATEO ⁻ ₍₆₋₁₎	0.5	0.49
		1.0	0.35
		2.0	0.80
S	pXβCATEO ⁺	0.5	7.90
		1.0	8.47
		2.0	8.26

All transfections included 0.5 pmoles of pSVδRB to supply T/t antigen. In the scrapefections, the total amount of plasmid DNA was kept to a constant 2.5 pmoles with pUC18.

developmental controls (27,28). Not surprisingly therefore, the promoter on its own showed little basal activity even when linked to the SV40 enhancer (Fig. 2B, lanes 7–11). However, even in this cellular environment, replication still increased expression 9-fold (lane 12). This suggests that replication partially overcomes negative regulation of the human β globin promoter. It is worth re-iterating here that the amount of replication in these semi-permissive human cells is low, typically giving rise to a less than two-fold increase in template numbers. Thus, overcoming negative regulation in K562 cells is unlikely to be due to titration of a negative factor.

When the *Xenopus* β globin promoter constructs were transfected into mouse L-cells, which are non-permissive for SV40 replication (29,30), the activity of the construct containing the intact replication origin (Fig 2C, βEO⁺, lane 6) was no greater than that of the construct containing the mutant origin (βEO⁻, lane 4). Thus, in cells where the SV40 origin cannot initiate replication, constructs containing this sequence are not activated for expression. This result, in which replication was prevented by a non-permissive cellular environment, is in agreement with results from the experiments where replication was blocked either by the drug, araC, or by a mutation in the origin (10 and this study).

Even in the absence of replication, the presence of T-antigen led to elevated expression in L-cells (Fig 2C, compare lanes 2, 4 and 6 with 1, 3 and 5). Weaker but detectable levels of T-antigen transactivation were also observed in HeLa cells (Fig 1, compare '+' and '-' lanes for β and βEO⁻ constructs). Our failure to detect this in our previous report (10) was most likely a consequence of the reduced sensitivity of the RNA analysis used in that study compared to the CAT assays used here. Nevertheless, T-antigen transactivation is clearly distinct from stimulation by replication because not only do we observe T-antigen transactivation in the absence of replication in L-cells (Fig 2C), but we also observe stimulation of expression by replication in Daudi and K562 cells where no T-antigen transactivation takes place (Figs 2A and B).

The data presented so far show that even though the degrees of enhancement and transactivation of β globin promoters vary between cell types, replication dramatically stimulates expression in all permissive cells.

Before consideration of the mechanism by which replication stimulates expression, we were concerned to explore further the

Figure 3. Replication does not further stimulate a transactivated β globin promoter. A representative CAT assay comparing the activities of the Xenopus β globin promoter with (pX β CAT $_{ori}^{+}$) or without (pX β CAT, pX β CAT $_{ori}^{-}$) linkage to a functional SV40 origin of replication. Constructs were assayed both in the presence and absence of T antigen by cotransfection with pSVEO $^{+}$ (T antigen-negative) or pSV δ RB (T antigen-positive).

effect of transfection protocol on the magnitude of that stimulation. The extent of induction of constructs introduced by calcium phosphate co-precipitation was only a few-fold in contrast to the induction by replication seen when constructs are transfected using DEAE-dextran (10 to 100-fold) (10 and this study). To further explore the effect of transfection protocol, we investigated two additional methods: electroporation and a modified scrape loading protocol or 'scrapefection' (31). Both methods gave rise to a significant replication enhancement in HeLa cells, amounting to 37-fold and 10–25 fold respectively (Table 1). Calcium phosphate co-precipitation therefore appears to exhibit the weakest replication effect of the four protocols tested, possibly because of the high level of expression without replication. Whether this is due to the time within the cell cycle when templates enter the nucleus (32) or the swamping of cells with calcium ions (33) is currently unknown.

Replication facilitates enhancer action

The highest levels of expression from the β globin promoters in our experiments have been achieved by a combination of replication, enhancement and transactivation by T-antigen. To identify the step in gene activation that is facilitated by replication, we determined separately the effects of replication on enhancement and transactivation. To do this we built additional β CAT constructs lacking the enhancer but containing either the mutant (ori^{-}) or wild type (ori^{+}) origin of replication. By comparing the activities of these constructs, in the presence or absence of T-antigen, with the parental clone pX β CAT, we were able to determine whether replication facilitates transactivation (Fig 3). The presence of either origin cassette (lanes 3–6) had little influence on the activity of the promoter (lanes 1 and 2), either in the presence or absence of T-antigen. In particular, the extent of transactivation by T-antigen (lanes 2 and 4) was not increased by replication of the construct (lane 6). In contrast, the dramatic stimulation of expression from the enhancer-containing construct (lane 8) indicates that replication acts specifically upon the process of enhancement.

Figure 4. Replication dependence of the SV40 early and adenovirus 5 E1A transcription units. (A) Representative histogram showing the levels of activity of the SV40 early promoter linked to a functional (pB/STSV EO^{+} CAT) (lanes 1, 2) or non-functional (pB/STSV EO^{-} (6-1)CAT) (lanes 3, 4) origin of replication. Constructs were transfected into HeLa cells with (stippled bar) or without (open bar) the T antigen-producing plasmid, pSV δ RB. (B) Primer extension analysis of E1A transcription. 0.5 pmoles each of pXSTOP, which contains the adenovirus 5 E1A gene (lanes 1, 2, 5, and 6) or pSVXL3 in which the E1A gene is linked to the SV40 origin of replication (lanes 3, 4, 7, and 8) were transfected in duplicate into HeLa cells either without (lanes 1–4) or with (lanes 5–8) pSV δ RB which provides T antigen. In both constructs the E1A transcription units have been mutated so that functional E1A proteins are not synthesized (16,17). The expected length of extension product from correctly initiated transcripts is ~113 nucleotides (indicated by arrow). The markers (M) are 32 P-labelled pUC18 (left) and pAT153 (right) digested with HpaII, and sizes are given in base pairs.

Are there enhancer-promoter combinations which are fully active without replication?

Thus far we have described the effects of replication only on SV40 enhancer/ β globin promoter combinations. In view of the fact that the SV40 viral genome undergoes a switch in gene expression coincident with replication, it was important to determine if the replication stimulation we observe is related to the viral early to late switch or is a more general phenomenon. To examine this point, we determined the effect of replication on expression from viral enhancer/promoter combinations active prior to replication of the viral genome.

(a) *The SV40 early transcription unit.* The SV40 early promoter linked in its natural position to the SV40 enhancer is active before the onset of replication during a viral infection, and therefore

Figure 5. The effect of replication on transcription from retroviral LTRs. **(Left)** Reporter plasmids containing the Rous sarcoma virus or Murine sarcoma virus long terminal repeats (LTRs) linked to the CAT gene and cassettes containing the wild type or mutant SV40 origins of replication are indicated diagrammatically. **(Right)** CAT analysis. Each construct (0.5 pmoles) was transfected into HeLa cells along with 0.3 pmoles pSV δ RB (+ T antigen) or 0.3 pmoles pSVEO⁺ (- T antigen).

it might not be expected to be activated by replication. SV40 early promoter constructs containing the mutant or wild type origin of replication were therefore tested in HeLa cells (Fig 4A). Expression from the construct containing the intact origin of replication was stimulated 47-fold in the presence of T-antigen (compare lanes 1 and 2). In contrast, the construct containing the mutant origin was unstimulated in the presence of T-antigen (compare lanes 3 and 4). Thus it appears that enhancement of even the SV40 early promoter is stimulated dramatically by replication in these transient transfections. Our previous report that this was not the case was based on immunofluorescence data on single cells (10). The number of expressing (fluorescing) cells was not significantly greater for transfections with replicating versus non-replicating constructs. Taken together, this study and our previous work suggest that sufficient templates to generate a fluorescent signal are activated in each cell even without replication, but that replication greatly elevates either the number of active templates per cell or the efficiency of transcription from individual templates.

(b) Adenovirus E1A gene. Another viral enhancer-promoter combination active prior to replication of the viral genome during infection, is the adenovirus E1A transcription unit. In addition to extending our observations to an enhancer other than SV40, we were interested to re-investigate the effect of replication on transcription of this gene because, like the SV40 early region discussed above, E1A expression has been reported to be unaffected by replication in transient assays (34). In particular, the autoregulatory activity of E1A proteins (35) might have masked any replication effect by upregulating the promoter in the absence of replication.

We therefore employed two constructs (pXSTOP and pSVXL3) containing E1A coding sequences mutated to prevent autoregulation (16,17), one of which (pSVXL3) also carried the

SV40 origin of replication. These constructs were transfected into HeLa cells in the presence or absence of T-antigen to activate the origin. Primer extension RNA analyses from duplicate transfections are presented in Figure 4B. T-antigen transactivation of this gene was low (faint signals in lanes 5 and 6). However, in contrast to the previous report where non-mutant E1A was used (34), replication stimulated E1A transcription dramatically (lanes 7 and 8, 45 to 71-fold over several experiments). Thus, when autoregulation by E1A is prevented, this enhancer-promoter combination is also subject to dramatic replication stimulation. **(c) Retroviral long terminal repeats.** As a further test of the generality of the replication effect, we analysed two retrovirus long terminal repeats (LTRs), which contain enhancers and their cognate promoters. Retroviruses are not thought to undergo an early to late switch coupled to replication. The relative levels of CAT produced by pRSVCAT (14) and derivatives containing the mutant or wild type origin, or pMSVCAT (36) and equivalent derivatives, are shown in Figure 5. Both LTRs were transactivated by T antigen some 3 to 10 fold. However, replication increased CAT activities relative to the transactivated LTRs more than 30-fold for the RSV LTR and 9-fold for the MSV LTR (Fig 5, compare lane 6 with lanes 2 and 4 or lane 13 with lanes 9 and 11). These findings further demonstrate that a broad range of enhancer/promoter combinations can be stimulated by DNA replication, and that this behaviour is not restricted to elements from DNA viruses which undergo a replication-induced switch in programmed gene expression.

Identification of a minimal enhancer capable of synergy with DNA replication

The enhancers employed in the previous sections are relatively complex elements which (*in vitro* at least) bind a large and heterogeneous collection of transcription factors. Their

Figure 6. Xenopus β globin promoter/SV40 enhancer/origin constructs, including those involving synthetic enhancers based on reiteration of enhancer motifs from the SV40 enhancer. (A) Schematic representation of pX β CAT derivatives in which various SV40 based enhancer and/or origin cassettes were placed downstream of the CAT gene. The intact enhancer and origin cassettes are drawn as boxes, with a filled triangle representing the approximate location of the ori core. Synthetic enhancer multimers are shown as arrow heads. The large box adjacent to the CAT gene contains the SV40 small t intron and polyadenylation site. (B) The arrangement of enhancer elements within a single copy of the SV40 72 bp repeats and adjacent (late side) sequences. The enhancer elements (A, B, C) consist of paired enhancers (Core A, Core C, Sph I and II). (C) Sequences of the multimerized repeats. Each repeat consists of paired sites for factors which bind to the SV40 enhancer. The two halves of the XhoI restriction site produced by reiteration of the oligonucleotides are boxed. All synthetic enhancers except C16 are in the (–) orientation so that when reading left to right, the sequences as shown here point through the CAT gene towards the promoter. Core C differs from core A by a single T to A transversion (circled). The two point mutations in the SphI motif, corresponding to the dpm2 mutation, are circled. The TC-II motif is identical to the kappa B motif found in many viral and cellular enhancers (for references see 37).

complexity and general lack of similarity to each other argues against a single factor which is correlated with replication dependency. Instead it seems more probable that the action of each enhancer as a whole is the target for modulation by replication. To test this, we examined whether less complex enhancers, generated by reiteration of individual protein binding

Figure 7. Synergism between synthetic enhancers and DNA replication in HeLa cells. Representative CAT assays comparing the activities, in transfected HeLa cells, of the Xenopus β globin promoter linked to synthetic enhancers produced by multimerization of wild type and mutant enhancer motifs. Each reporter construct was cotransfected with pSV δ RB to supply T antigen.

sites (enhancers) taken from the SV40 enhancer, would also be replication-dependent. The SV40 enhancer is composed of at least three discrete 15–20 bp elements (A, B and C; Fig 6B) which cooperate with one another or with duplicates of themselves to enhance transcription (38). These units are distinct from the individual protein binding sites (enhancers) of which they are comprised. Several multimerized synthetic copies of enhancer motifs have been shown to act in HeLa cells as strong enhancers of mammalian β globin promoters (12,13,39). We placed six copies of motifs from each of the three enhancer elements A, B and C immediately downstream of the CAT gene cassette in each of pX β CAT, pX β CATori[–](6-1) and pX β CATori⁺ (Fig 6A and 6C). The point mutants (core A/core C and dpm2) which inactivate the A and B elements respectively were also tested.

The activities of the synthetic enhancers coupled to the Xenopus β globin promoter with no origin, the wild type origin or the mutant origin were compared to the activities of the full length

enhancer contained within the $EO^{-}_{(6-1)}$ and EO^{+} cassettes. The multimers previously shown by Ondek *et al.* (12) to be inactive as enhancers (core A/core C and *dpm2*) were unable to stimulate the *Xenopus* β globin promoter even when replicated (Fig. 7, lanes 7–9 and 13–15). The other multimers, core A/core A (lanes 4–6), B20wt (lanes 10–12) and C16 (lanes 16–18), increased transcription in response to replication by an amount (20 to 30-fold) equivalent to the full length enhancer (lanes 1–3). This indicates that replication can cooperate with enhancers consisting only of multiple copies of one or two types of enhancer motif. Point mutations which inactivate these enhancers also prevent stimulation through replication. It therefore seems unlikely that enhancers contain specific elements which are regulated by replication, and that instead replication cooperates with enhancers as a whole. Replication therefore seems to be a general mechanism by which enhancer action is facilitated in transient assays.

DISCUSSION

Replication facilitates enhancement

The data presented in this paper demonstrate a general role for DNA replication in the activation of gene transcription in transient transfection assays. In our earlier studies we excluded both covalent modifications of the template, such as methylation, and gross copy number increase as the explanation for replication-induced stimulation of gene expression (10). In the experiments reported here, the semi-permissive nature of human cells meant that the overall increase in template copy number due to replication was less than two-fold, nevertheless stimulation of expression by one or two orders of magnitude was routine. Although it is difficult to formally exclude the possibility that the copy number of a subpopulation of templates increased preferentially, this is unlikely to explain the specificity of the replication effect for enhanced templates (see below), since we and others (40) have shown that the amount of replication per cell, from an SV40 origin, is equivalent for enhanced and unenhanced templates (see also 41).

Using RNA analysis, we were previously unable to detect either transactivation by T-antigen or enhancement in the absence of replication (10). However, using the sensitive CAT assay, we show here that transactivation and enhancement in the absence of replication do indeed occur, albeit to differing degrees in different cell types. Importantly, though, in all the cell types tested, where the SV40 origin was active, dramatic stimulation of expression by replication was always observed.

The detection of transactivation and enhancement in the absence of replication raised the question of which of the two activation cues is potentiated by replication. The mechanism of T-antigen transactivation is currently unclear but likely to include modifications of transcription factors and possibly direct involvement of T-antigen in the initiation complex. Whatever the mechanism, though, we show here that transactivation is not increased by replication. Consistent with this observation, the level of transactivation that we detect in these DEAE dextran-mediated transfections is very similar to that found using calcium phosphate coprecipitation (42), which supports only a small replication effect (10). Thus, since transactivation is not potentiated by replication, the dramatic increase in activity that we observe must be due to facilitation of enhancement.

The potentiation of enhancement is not restricted to any particular enhancer-promoter combination. In addition to the human and *Xenopus* β globin genes reported here, we have

observed a strong effect of replication on the human α globin gene linked to the SV40 enhancer (data not shown). The earlier report that human β globin gene expression is not stimulated by DNA replication was deduced from calcium phosphate transfections (24), which unlike the three methods described here, support only a small replication effect (10). Other genes previously reported not to be replication inducible, even in DEAE dextran transfections, such as the SV40 early transcription unit (10) and the adenovirus E1A gene (34), have been shown here to be dramatically stimulated. In infected cells, these two transcription units are both expressed prior to replication of the viral genome, making it unlikely that the replication effect we observe is restricted to the viral early to late switch in gene expression. This conclusion is reinforced by our observation that retroviral LTRs can also be stimulated by replication. These viruses do not undergo an early to late switch coupled to replication. Similar observations made for the human immunodeficiency virus (HIV) have led to the suggestion that replication of the host genome in response to a mitogenic signal may stimulate transcription of the provirus (43).

What is the mechanism by which replication facilitates enhancer action?

Two potential mechanisms, the relief of promoter occlusion and the loading of a specific enhancer factor, are unlikely on current evidence. Replication-dependent activation of the adenovirus pIX gene is thought to reflect the release of promoter occlusion caused by readthrough from the E1B gene promoter which is immediately upstream (44). This is a specialised situation and unlikely to occur in all of the enhancer-promoter combinations tested in this study. It also seems unlikely that replication acts by recruiting a specific enhancer-binding protein based on the diversity of natural and synthetic enhancers used in this study, all of which respond to replication.

A plausible mechanism for the action of replication on transfected templates may be through chromatin remodelling. Studies of chromatin assembly in *Xenopus* egg extracts suggest that assembly at the replication fork differs from that on duplex DNA in terms of the kinetics of the assembly process and perhaps also the biochemical composition of the resulting chromatin (45,46). Assembly of nucleosomes onto newly transfected double-stranded plasmids may likewise differ from assembly during replication. There is considerable evidence for a competitive relationship between transcription complex assembly and chromatin assembly (reviewed in 7). The inhibitory contribution of chromatin can range from a relatively subtle masking of a transcription factor binding site by a positioned nucleosome core to the formation of a highly compacted and inaccessible structure including histone H1. In a simple scheme, chromatin formation in the absence of replication may be biased against the assembly of transcription factor complexes involved in enhancer-mediated activation. Transcription factors probably have access to the core promoter since the β globin promoter in our experiments has basal activity and can be transactivated by T-antigen. Furthermore, transcription from the promoter-only constructs is not increased by replication, which might have been expected if nucleosomal repression was taking place. The SV40 enhancer may also be accessible to transcription factors without replication since it is nuclease hypersensitive before replication in DEAE dextran-mediated transfections (47).

Suggestions for how chromatin might specifically inhibit enhancer action can only be speculative, particularly since the mechanism for action at a distance is itself unclear. The assembly

process might affect, for example, the extent of histone modifications, the distribution of histone H1, the precise spacing of nucleosomes and the composition of non-histone proteins, and these in turn may influence communication between transcription factors associated with enhancers and core promoters. A more detailed understanding of the different chromatin assembly processes is likely to be informative in this regard.

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REFERENCES

- Holmquist, G.P. (1987) *Am. J. Hum. Genet.* **40**, 151–173.
- Groudine, M. and Weintraub, H. (1982) *Cell* **30**, 131–139.
- Holtzer, H., Weintraub, H., Mayne, R. and Mochas, B. (1972) *Curr. Top. Dev. Biol.* **7**, 229–256.
- Humphries, E.H. and Temin, H.M. (1974) *J. Virol.* **14**, 531–546.
- Miller, A.M. and Nasmyth, K. (1984) *Nature* **312**, 247–251.
- Thomas, G.P. and Mathews, M.B. (1980) *Cell* **22**, 523–533.
- Felsenfeld, G. (1992) *Nature* **355**, 219–224.
- Svaren, J. and Chalkley, R. (1990) *TIGS* **6**, 52–56.
- Wolffe, A.P. and Brown, D.D. (1986) *Cell* **47**, 217–227.
- Enver, T., Brewer, A.C. and Patient, R.K. (1988) *Mol. Cell. Biol.* **8**, 1301–1308.
- Gorman, C.M., Moffat, L.F. and Howard, B. (1982) *Mol. Cell. Biol.* **2**, 1044–1051.
- Ondek, B., Gloss, L. and Herr, W. (1988) *Nature* **333**, 35–45.
- Tanaka, M., Grossniklaus, U., Herr, W. and Hernandez, N. (1988) *Genes Dev.* **2**, 1764–1778.
- Gorman, C.M., Merlino, G.T., Willingham, M.C., Pastan, I. and Howard, B.H. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 6777–6781.
- Gorman, C.M., Rigby, P.W.J. and Lane, D.P. (1985) *Cell* **42**, 519–526.
- Smith, D.H., Kegler, D.M. and Ziff, E.B. (1985) *Mol. Cell. Biol.* **5**, 2684–2696.
- Schneider, J.F., Fisher, F., Goding, C.R. and Jones, N.C. (1987) *EMBO J.* **6**, 2053–2060.
- Gorman, C.M. 1985 in Glover, D.M. (ed.) *DNA Cloning volume II: a practical approach*. IRL Press, Oxford. p143–190.
- Wilson, A.C. and Patient R.K. (1990) in Murray, E.J., and Walker, J.M. (eds.) *Methods in Molecular Biology*, vol 7: Gene expression and transfection techniques. Humana Press Inc, Clifton NJ. Chp 29, pp 397–404.
- Eustis-Turf, E., Wang, K-M. and Schook, L.B. (1987) *BioRad Laboratories Bulletin* **1347**.
- Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159.
- Leonard, M.W. and Patient R.K. (1990) in Murray, E.J., and Walker, J.M. (eds.) *Methods in Molecular Biology*, vol 7: Gene expression and transfection techniques. Humana Press Inc, Clifton NJ., pp 297–306.
- Crabb, D.W. and Dixon, J.E. (1987) *Anal. Biochem.* **163**, 88–92.
- Treisman, R., Green, M.R. and Maniatis, T. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 7428–7432.
- Gluzman, Y., Sambrook, J.F. and Frisque, R.J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3898–3902.
- Gluzman, Y. (1981) *Cell* **23**, 175–182.
- Charnay, P. and Maniatis, T. (1983) *Science* **220**, 1281–1283.
- Dean, A., Ley, T.J., Humphries, R.K., Fordis, C.M.Jr., Nienhuis, A.W. and Schechter, A.H. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 5515–5519.
- Braithwaite, A.W., Sturzbecher, H-W., Addison, C., Palmer, C., Rudge, K. and Jenkins, J.R. (1987) *Nature* **329**, 458–460.
- Gerard, R.D., Guggenheimer, R.A. and Gluzman, Y. (1987) *J. Virol* **61**, 851–857.
- McNeil, P.L., Murphy, R.F., Lanni, F. and Taylor, D.L. (1984) *J. Cell Biol.* **98**, 1556–1564.
- Nicolau, C. and Sene, C. (1982) *Biochim. Biophys. Acta* **721**, 185–190.
- Pine, R., Levy, D.E., Reich, N. and Darnell, J.E.Jr. (1988) *Nucl. Acids Res.* **16**, 1371–1378.
- Matsui, T., Murayama, M. and Mita, T. (1986) *Mol. Cell. Biol.* **6**, 4149–4154.
- Nevins, J.R. (1986) *CRC Crit. Rev. Biochem.* **19**, 307.
- Laimins, L.A., Khoury, G., Gorman, C., Howard, B. and Gruss, P. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 6453–6457.
- Phares, W., Franza, B.R. and Herr, W. (1992) *J. Virology* **66**, 7490–7498.
- Herr, W. and Clarke, J. (1986) *Cell* **45**, 461–470.
- Fromental, C., Kanno, M., Nomiyama, H. and Chambon, P. (1988) *Cell* **54**, 943–953.
- Weintraub, H. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5819–5823.
- de Villiers, J., Schaffner, W., Tyndall, C., Lupton, S. and Kamen, R. (1984) *Nature* **312**, 242–246.
- Cao, S.X., Mishoe, H., Elion, J., Berg, P.E. and Schechter, A.N. (1989) *Biochem. J.* **258**, 769–776.
- Proudfoot, N.J., Lee, B.A. and Monks, J. (1992) *New Biologist* **4**, 369–381.
- Vales, L.D. and Darnell, J.E.Jr (1989) *Genes Dev.* **3**, 49–59.
- Wolffe, A.P. (1991) *J. Cell Sci.* **99**, 201–206.
- Almouzni, G., Mechali, M. and Wolffe, A.P. (1990) *EMBO J.* **9**, 573–582.
- Cereghini, S., and Yaniv, M. 1984 *EMBO J.* **3**:1243–1253.