Activation of the adenovirus 2 protein IX promoter by DNA replication in a transient expression assay

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ABSTRACT

We have studied the effect of template replication on transcriptional activation of the promoters for the adenoviral protein IX and Ela genes using a short-term transient expression assay. DNA replication mediated modulation of the transcriptional activity of these promoters was monitored using plasmids with limited replication capability conferred by the SV40 minimal origin of DNA replication in the monkey kidney cell line, COS M6. Our results indicate a substantial increase in protein IX promoter directed transcription in a dosage-independent manner as a consequence of the DNA replication process. In contrast, the transcriptional activity of the adenovirus 2 Ela promoter was not significantly altered. In addition to this DNA replication-mediated effect, transcription from the protein IX promoter was positively regulated by the adenovirus Ela 13S and E4 gene products but was repressed by the Ela 12S gene product to a limited extent. The evidence suggests that the effect of template replication on the transcriptional activity of plasmid-borne protein IX and Ela gene promoters in this transient expression system closely mimics the situation on the viral chromosome.

INTRODUCTION

The expression of some cellular genes appears to be temporally and functionally coupled to DNA replication, as illustrated by the cell-cycle dependent regulation of histone gene expression (1). Indeed, redirections of transcriptional activity observed during developmental programming is, in many instances, associated with DNA replication (2). It appears, therefore, that RNA polymerase II may initiate transcription by mechanisms mediated by DNA synthesis.

Analogous alterations of transcriptional activity are observed during the early to late transition in the life cycle of DNA tumor viruses, such as that observed during productive infection by human adenoviruses. Lytic infection by adenovirus proceeds through two broadly defined phases, early and late, punctuated by the onset of DNA replication (3). It is not known whether template replication has a stimulatory effect on transcriptional initiation at the major late promoter, since the late promoter is active at early times and

the abundance of late mRNAs after replication may merely reflect an increased availability of viral templates for transcription (4). However, as shown by Thomas and Matthews (5), expression of the L4 and L5 regions of the late transcription unit is clearly mediated by a change in the physical state of the template conferred by DNA replication; the expression of these regions in the absence of DNA replication is prevented by progressive termination of transcription across the L2-L3 region (6). Expression of protein IX mRNA occurs exclusively after initiation of DNA replication and is completely abolished by hydroxyurea-induced inhibition of DNA synthesis (7). The protein IX promoter, therefore, serves as a good model for the analysis of DNA replication mediated activation of a RNA polymerase II promoter.

The initial analyses of the role of template replication on late gene (5) and protein IX (7) expression necessitated the use of complicated superinfection protocols initially developed by Bruner and Cape (8) in their investigations on T4 gene expression. We have, in this study, developed a simple and convenient transient assay system that permits facile analyses of the role of DNA replication on protein IX expression. For this purpose, we have constructed a chimeric gene in which the protein IX promoter has been fused to a marker gene coding for chloramphenicol acetyltransferase (CAT). Insertion of the SV40 minimal origin of DNA replication (9, 10, 11) into this fusion plasmid permits its restricted replication in COS cells that constitutively express the SV40 large T antigen (12). Our results indicate that DNA replication plays an important role in the activation of protein IX promoter directed transcription in the transient assay system. The DNA replication mediated increase of protein IX promoter activity could be further augmented by viral coded trans-acting factors.

MATERIALS AND METHODS

Cell lines and culture.

HeLa and COS M6 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. COS M6 is a subclone of COS 1 cells (12) and was generously provided by P. Jat and P.A. Sharp.

Plasmids.

For construction of the protein IX promoter-chloramphenicol acetyltransferase (CAT) gene fusion plasmid, pIX-CAT, an 834 bp HindIII-SstI fragment (mp 7.8-10.1 on the Ad2 genome, Fig. 1A), blunt-ended at the SstI site and carrying the protein IX promoter, was isolated from plasmid pGC341 (13) which contains the Ad2 XhoIC fragment (mp 0-16.0). This fragment was cloned between the HindIII and SmaI sites in the polylinker of pUC-CAT (14) to yield pIX-CAT.

Fig. 1. Organization of the adenovirus 2 ELB transcription unit and construction of recombinant plasmids. A, mRNAs from the ELB region (45). The larger mRNAs (13S to 22S) are expressed at early times. The 9S mRNA is expressed at late times and codes for protein IX. The DNA sequences are numbered according to Gingeras et al. (46). The TATA box for protein IX mRNA is at nucleotide position 3546 and transcription starts at position 3575 or 3577. B, Construction of IX-CAT plasmids. A HindIII-SstI fragment, containing the protein IX promoter, was inserted into the polylinker cloning site of pUC-CAT (see Materials and Methods). pIX ori-CAT was obtained by cloning the SV40 minimal origin of DNA replication into the unique BamHI site of pIX-CAT. The nucleotide numbering system for the SV40 minimal origin is as described in (9).

A replicating version of this plasmid in COS cells, pIX ori-CAT, was obtained by inserting a 100 bp BamHI fragment carrying the SV40 minimal origin of DNA replication (9) into the unique BamHI site of pIX-CAT (Fig. 1B). The methylation minus plasmid, pIX ori-CAT dam^- , was obtained by propagation of pIX ori-CAT in a dam^- strain of *E. coli*, GM119. pBL209-ori 1 and pBL209-ori 2

were constructed by insertion of the SV40 minimal origin containing BamHI fragment, in either orientation, into the unique BamHI site of pBL209, an Ad5 XhoIC fragment containing plasmid (15). Plasmid pGC212, containing the left-most 8% of the Ad2 genome, has been described (13). pC831 and p12S are plasmids respectively expressing the Ela 13S and 12S gene products alone (16, 17). pE4, expressing the E4 mRNAs, contains the Ad2 EcoRIC fragment cloned in pBR322.

DNA transfections.

Transfections of COS and HeLa cells were carried out using the calcium-phosphate precipitation method (18). Briefly, 20 µg of plasmid DNA in 1 ml was added, as a calcium-phosphate co-precipitate, to 2×10^6 cells plated on a 100 mm dish. After incubation for 10 hours, the cells were rinsed twice with PBS, fresh medium added and incubation was continued for an additional 38 hours. In co-transfection experiments, a 3:1 molar ratio of trans-regulating to test plasmid (5 µg of test plasmid and 15 µg of trans-regulating plasmid) was used. The final amount of plasmid DNAs was brought to 20 µg/ml with pUC-DNA, when required. Where applicable, cytosine arabinoside was added at 25 µg/ml after transfection.

CAT assays.

CAT enzyme levels in transfected cells were measured as described (19) using equal volumes of cell extracts. The results were quantitated by scintillation counting of the product spots (acetylated chloramphenicol) in the chromatograms.

Replication analysis in COS cells.

Low-molecular weight DNA was prepared from transfected cells as described (20). DNA samples were treated with DNase-free RNase, digested with either DpnI or MboI, fractionated on a 1.2% agarose gel and transferred to nitro-cellulose filters (21) for hybridization with nick-translated ^{32}P -labeled pUC-CAT DNA.

RNA isolation and S1 analysis.

Cytoplasmic RNA from COS cells was prepared by lysis in hypotonic buffer RSB (22) containing 0.5% NP-40 and SDS phenol-chloroform extraction of the supernatant after pelleting of nuclei. Precipitated nucleic acids were treated with 25 U per ml of RQ1 DNase (Promega Biotec) in DNase digestion buffer in the presence of 50 U of RNasin at 37°C for 20 min, followed by phenol extraction and ethanol precipitation. S1 nuclease analysis of CAT and Ela mRNAs was performed as described by Weaver and Weissman (23). Approximately 10^5 cpm of end-labeled DNA probe was annealed to 30 µg of cytoplasmic

RNA at 53°C for 12-16 hours followed by digestion of hybrids with S1 nuclease (Sigma Chemical Co.) at 1000 U per ml at 20°C for 1 hr. S1-digested products were analyzed by fractionation on 6% acrylamide-8M urea gels. For analysis of CAT RNA, a 1114 bp EcoRI-HindIII fragment (Fig. 1B), 5' end-labeled at the EcoRI site in the CAT gene was used. Ela-specific mRNAs were quantitated with a 714 bp PvuII-XbaI fragment (mp 1.7-3.7 on the Ad5 genome), 5' end-labeled at the XbaI site. 5' end-labelings were carried out with T4 kinase and γ -³²P ATP (3000 Ci/mmol, NEN).

RESULTS

DNA replication and Ad2 gene products activate the protein IX promoter

In order to identify the viral functions that are required for activation of the protein IX gene promoter, we initially studied the expression of pIX-CAT in HeLa cells with and without Ad2 infection. These studies revealed an enhancement of the IX promoter directed CAT expression upon Ad2 infection (results not shown) suggesting a role for virus-induced transacting factors in the activation of protein IX gene expression. These results were also faithfully duplicated in COS M6 cells (see below). The COS cells, therefore, constitute an adequate system for analysis of the total effects of DNA replication and transacting factors on IX-CAT expression.

Transfection of COS cells with pIX-CAT resulted in a low basal level of CAT expression (Fig. 2A, lane 4); the level of CAT expression was increased by about 3-4 fold when the transfected cells were infected with Ad2 (Fig. 2A, lane 5). Inclusion of the SV40 minimal origin of DNA replication in pIX-CAT (pIX ori-CAT) resulted in a consistent 6-10 fold increase in CAT activity (Fig. 2A, lane 6). The CAT activity of pIX ori-CAT, however, diminished to levels directed by pIX-CAT in the presence of DNA synthesis inhibitor, cytosine arabinoside (Fig. 2A, lane 8) indicating that plasmid DNA replication results in substantial stimulation of IX-CAT expression. To determine if the level of CAT expression of pIX ori-CAT could be further augmented by viral coded transacting regulatory factors, cells transfected with pIX ori-CAT were infected with Ad2. As seen in Fig. 2A, lane 7, viral infection resulted in a 2-4 fold stimulation of CAT expression. The extent of protein IX promoter stimulation upon adenovirus infection of transfected cells, although measuring only 2-4 fold, is significant and has been consistently observed in four different experiments.

To rule out any possibility of the non-methylation of newly replicated plasmid DNA being responsible for the increased activity of pIX ori-CAT, we

Fig. 2. Effect of DNA replication, Ad2 infection and methylation on IX-CAT expression in COS M6 cells. The acetylated forms of chloramphenicol are marked by arrows; CM indicates unreacted chloramphenicol. A, CAT activity in COS cells transfected with 20 μ g of pRSV-CAT (lane 3), pIX-CAT (lanes 4, 5) and pIX ori-CAT (lanes 6, 7, 8). In lanes 5 and 7, cells were infected with Ad2 24 hours before harvest. In the assay shown in lane 8, cytosine arabinoside (25 μ g/ml) was added immediately after the transfection. Lane 1, marker reaction with commercial CAT enzyme; lane 2, mock transfection. B, Effect of methylation on IX-CAT expression. CAT activity in transfections of pIX ori-CAT (lane 1) and pIX ori-CAT dam^+ (lane 2) in COS M6 cells.

compared the levels of CAT expression directed by pIX ori-CAT and the methylation minus plasmid, pIX ori-CAT dam^+ and observed no significant change in the activity of the IX promoter in either plasmid (Fig. 2B). Our observation is also supported by findings of Kruczek and Doerfler (24) that methylation of sequences adjacent to the Ad12 protein IX promoter does not affect transcription.

Our results, therefore, suggest a dominant role for DNA replication in transcriptional stimulation of the IX promoter. Furthermore, they suggest that promoter stimulation may be maximized by trans-acting adenoviral gene products.

Cytoplasmic RNA levels of protein IX promoter activation

To establish that the increase in IX-CAT activity upon DNA replication was due to elevated levels of CAT mRNA, we measured steady-state levels of IX-CAT mRNA by quantitative S1 nuclease mapping. The 5' end-labeled probe (see Materials and Methods for description), which measures the cap site of

Fig. 3. Cytoplasmic RNA analysis of IX promoter activation by DNA replication. Quantitative S1 nuclease analysis of CAT mRNAs from COS cells transfected with pBR322 (lane 1), pIX-CAT (lane 2), pIX ori - CAT (lane 3) and pIX ori - CAT in the presence of ara C (lane 4); M, molecular weight marker.

the IX-CAT chimeric transcript, should yield an S1-protected DNA fragment of 305 bases since the transcriptional initiation site for the protein IX promoter has been mapped to mp 9.9 on the Ad2 genome (25). As seen in Fig. 3 (lanes 2-4) a fragment of 305 bases was readily detected. In addition, a fragment of higher molecular weight was also protected in our S1 analyses; this could possibly be accounted for by the 5' terminal heterogeneity reported for protein IX mRNA (25). Consistent with the levels of CAT activity described above, we have observed a 5-7 fold increase (in three different experiments) in IX-CAT mRNA levels upon template replication (Fig. 3, lane 3). Activation of IX-CAT expression by DNA replication is independent of template number

It is possible that the above results on replication-mediated stimulation of IX promoter directed transcription could be caused by an increase in gene dosage due to plasmid replication in COS cells. We have, therefore, assayed the replicative efficiency of IX-CAT recombinants in COS cells by restriction

Fig. 4. Replication of IX-CAT recombinants in COS M6 cells. Low molecular weight DNAs from COS cell-transfections of pIX-CAT (1), pIX ori-CAT (2), and pIX ori-CAT in the presence of ara C (3) were fractionated, undigested or digested with DpnI or MboI, on a 1.2% agarose gel, transferred to nitrocellulose and probed with nick-translated ^{32}P -labeled pUC-CAT DNA. The positions of the linear(L), relaxed circular (R), and supercoiled (S) forms of plasmid DNAs are indicated.

analysis, prior to Southern blotting, of low-molecular weight DNAs from various transfections with DpnI and MboI. DpnI and MboI cleave at the same recognition sequence GATC; however, DpnI only recognizes methylated adenosine residues, whereas MboI cleaves at the non-methylated sequence. MboI-resistant plasmid DNAs, therefore, constitute input methylated DNA (of prokaryotic origin) whereas DpnI-resistant DNA represents newly-synthesized plasmid DNA in COS cells since mammalian cells lack an adenosine methylase. It is clear from a comparison of DpnI- and MboI-resistant IX-CAT DNAs (Fig. 4) that there is only a marginal increase in gene dosage due to the replication process. In fact, in different assays, the amount of newly synthesized DNA (induced by replication of the SV40 minimal origin) barely exceeded one-half of input plasmid DNA in a 48 hour transient expression assay. This is consistent with the observation that plasmids carrying this 100 bp SV40 minimal origin replicate very poorly compared to those bearing the wild type origin (9). It is

possible that quantitation of plasmid DNAs in the Hirt supernatant may not accurately reflect the number of transcriptionally active nuclear templates. However, the fact that transcription from the IX promoter is disproportionately large compared to that emanating from other adenoviral promoters borne on plasmids that replicate to the same extent as the IX-CAT plasmid (see below) seems to argue strongly against this possibility.

Effect of DNA replication on Ela expression

To establish that the amplification of transcription from the IX promoter upon plasmid replication is a promoter-specific event and is not reflective of a phenomenon true for all promoters due to some peculiarity of the system employed, we have examined the effect of DNA replication on transcription of the Ela gene contained in the plasmid pBL209 by quantitative S1 mapping of steady-state cytoplasmic RNA (Fig. 5). The Ela specific DNA probe, 5' end-labeled at the XbaI site (nucleotide position 1336 on the Ad5 genome), should yield an S1-protected DNA fragment of 110 bp (the 3' splice site for Ela RNAs being at nucleotide position 1226). Our results indicate a very marginal increase (about one-half) in Ela transcription upon plasmid replication (Fig. 5, lanes 3, 4) which can be readily accounted for by the slight increase in the amount of template DNA due to replication of the SV40 minimal origin in COS cells. We have observed no significant changes in the activity of the Elb promoter in plasmid pBL209 upon plasmid DNA replication (data not shown). The extent of plasmid replication induced by inclusion of the SV40 minimal origin in pBL209 was very similar to that observed for pIX ori-CAT. It is unlikely that the phenomenon of Ela-mediated enhancer repression (30, 31) affects our conclusions on the effect of DNA replication on the Ela promoter; plasmid pBL209 also expresses the Elb 19kd T antigen in the presence of which Ela-mediated enhancer repression does not occur (K. Yoshida and G. Chinnadurai, in preparation). Our results agree well with the observation that the increase in transcription from most early promoters upon viral DNA replication is probably reflective of an increase in the number of DNA templates (34). It appears, therefore, that the susceptibility of promoters to activation by DNA replication in this SV40 ori/COS cell system accurately reflects the situation on the viral chromosome.

Trans-regulation of IX-CAT expression

As indicated in Fig. 2A, Ad2 infection of pIX-CAT-transfected COS cells results in enhanced levels of CAT activity, suggesting that expression from the IX promoter is regulated by trans-acting viral gene products. The effect of infection of several Ad2 and Ad5 mutant viruses carrying lesions in the

Fig. 5. Effect of DNA replication on El α transcription. S1 nuclease mapping of El α -specific mRNAs from transfections of pBL209 (lane 2) pBL209-ori 1 (lane 3) and pBL209-ori 2 (lane 4). Lane 1, mock transfection; M, molecular weight marker, and P, probe.

various early genes (El α , Elb, E3, and E4) on IX-CAT expression was analyzed (data not shown) and the results obtained suggested a possible role for the El α and E4 genes in IX promoter regulation. We have, therefore, examined the effect of cloned viral early genes, namely the El α 12S and 13S and E4 gene products, on IX-CAT expression in co-transfection experiments. The El α 13S and 12S gene products are known to activate transcription of several viral and cellular genes carried on transfected plasmids (26, 27, 28, 29). Both 12S and 13S gene products suppress action of the SV40 enhancer (30, 31) whereas the 12S gene product alone has been reported to suppress transcription from the late promoter of the E2a gene (32). Also, E4 has been shown to stimulate transcription from the major E2a early start site (33).

Our results indicate that the trans-regulating effect of the Ela and E4 gene products on the CAT activity of both pIX-CAT and pIX ori-CAT was very similar (Fig. 6A). Whereas co-transfection of l2S resulted in a 2-3 fold decrease of IX promoter (lane 3) directed CAT activity, the l3S and E4 gene products stimulated CAT expression by 3-5 and 2-3 fold, (lanes 4, 5) respectively. This trans-acting effect of the Ela l2S and l3S as well as the E4 gene products on protein IX promoter directed transcription, although marginal, was consistently observed in four independent experiments and was not due to variations in transfection efficiency. The combined effect of the l2S and l3S gene products on pIX-CAT expression was additive, since co-transfection of pGC212 resulted in an only marginal increase of IX-CAT expression (Fig. 6A, lane 2). In agreement with this observation is our finding that infection of pIX-CAT transfected COS cells with an adenovirus mutant expressing the Ela l3S mRNA alone resulted in substantially higher levels of CAT activity than that observed with Ad2 *wt* infection. The combined effect of l3S and E4 co-transfection resulted in levels of CAT activity intermediate between that induced by the Ela l3S or E4 gene alone. It is possible that when both these trans-acting gene products are present, the Ela l3S gene product has the dominant trans-regulatory activity. The trans-acting effect of these adenoviral gene products on pIX ori-CAT expression although qualitatively similar was somewhat reduced suggesting that the dominant force governing protein IX promoter activity on a replicating template is the DNA replication event itself.

The stimulatory activity of the Ela l3S and E4 gene products described above was also reflected in quantitative S1-analysis of cytoplasmic RNA (Fig. 6B), suggesting that this trans-enhancement of CAT expression is due to increased steady-state levels of IX-CAT specific mRNA.

DISCUSSION

Our analysis of the role of DNA replication in transcriptional regulation of the protein IX and Ela promoters suggests that the transcription of the protein IX gene, but not that of Ela, is stimulated by DNA replication.

It also appears from our experiments that template replication may be the predominant force governing protein IX gene expression. As shown by Crossland and Raskas (7), the level of protein IX gene expression in the presence of hydroxyurea is barely perceptible. The low but significant levels of transcription directed by the IX promoter on a non-replicating plasmid may be reflective of transcription occurring on a circular rather than a linear

template such as the adenovirus chromosome; the expression of transfected DNA is apparently influenced by its topological state (35, 36), circular DNA molecules being more effective templates for transcription than linear DNA molecules. Alternatively, it may reflect a lack, in our experiments, of additional information (e.g. additional sequences or viral coded regulatory factors) normally required for stringent control of protein IX gene expression during the life cycle of adenovirus. It must be mentioned, however, that certain groups (37, 38) have found evidence for low level expression of protein IX in the presence of cytosine arabinoside, although this may have resulted from breakdown of the inhibitor of DNA synthesis during prolonged incubation (39).

The mechanism of DNA replication-mediated activation of the IX promoter is unclear. Our results indicate a substantial increase in protein IX promoter directed CAT mRNA levels as a consequence of the DNA replication process. We have found no evidence for direct activation of additional transcriptional start sites by DNA replication, such as that proposed for SV40 late gene expression (40). It is possible, then, that a replication-mediated alteration in template conformation or nucleosome distribution could facilitate increased entry of the transcriptional machinery at the protein IX promoter. In this regard, it is significant that micrococcal nuclease digestion of newly replicated viral DNA yields products that are distinctly different from those yielded by parental viral DNA (41). Alternatively, during or after replication, sequences that were previously inaccessible could become available for interaction with regulatory factors. In the latter case, it would appear from our experiments that a cellular transcriptional factor(s) may be involved.

A role for adenoviral early gene products in modulation of IX promoter-directed transcription is also evident from our experiments. Whereas the Ela 13S and E4 gene products possess a trans-activating function, it is significant that the Ela 12S gene product can repress 13S-mediated activation of the IX promoter. A similar viral encoded repressor may also be responsible

Fig. 6. Trans-regulation of IX-CAT expression. A, pIX-CAT or pIX ori-CAT was transfected alone, (lane 1) or with pGC212 (lane 2), pl2S (lane 3), pC831 (lane 4), pE4 (lane 5), and pC831+pE4 (lane 6). 3:1 molar ratios of trans-regulator to test plasmid were used. Where applicable, the final amount of plasmid DNAs was brought to 20 μ g with pUC DNA. Autoradiographic exposure for pIX-CAT was four times longer than that for pIX ori-CAT. B, Quantitative S1 mapping of IX-CAT RNA. M, molecular weight marker. Lanes 2-4: transfection of pIX-CAT alone (2), or with pC831 (3) or pE4 (4). Lanes 5-7: transfection of pIX ori-CAT alone (5), or with pC831 (6) or pE4 (7). Lane 1 represents a mock transfection.

for the inactivity of the IX promoter at early times of infection. It is not clear if any of these trans-regulators function by binding directly to sequences at or adjoining the promoter; in view of the relative lack of homology between different promoters activated by Ela, it has been suggested that the effect of Ela is probably mediated by a cellular transcriptional factor (42). Deletion analysis of sequences upstream from the IX-promoter should help resolve whether identical or dissimilar sequences are involved in mediation of the trans-acting functions of the Ela 12S and 13S and E4 gene products. Our experiments do not rule out any trans-acting function for the SV40 T antigen that is constitutively expressed in COS cells; however, as recently shown (43), endogenous T antigen does not influence E2 promoter activity in COS cells.

The use of the above described plasmid-based system for studying the effect of template replication on promoter regulation circumvents the need for stringent gene dosage controls and superinfection protocols previously employed to study this phenomenon. As recently shown (44), DNA replication is also required for maximal activation of the plasmid-borne US11 promoter of herpes simplex virus type 1. Since the submission of this manuscript, another paper (47) has appeared reporting substantially similar results on the effect of DNA replication on protein IX promoter activation. The fact that different viral promoters that are normally expressed only on replicating linear templates are also activated on replicating plasmids seems to argue for a general mechanism underlying the phenomenon of DNA replication mediated promoter activation. Deletion experiments on plasmid-borne promoters have led to the definition of the elements of eukaryotic promoters; similar analyses of promoters carried on replicating plasmids may unravel the mechanism of replication-mediated promoter activation.

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