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Adenovirus 2 early region 1A stimulates expression of both viral and cellular genes

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The ability of products from the adenovirus early region 1A to stimulate viral and cellular gene expression has been studied, using a transient expression assay in HeLa cells. We show that the E1A 13S mRNA encodes a diffusible product which is capable of stimulating transcription of adenovirus genes as well as the rabbit β-globin gene. The E1A 12S mRNA has no detectable stimulatory effect on either cellular or viral genes. Although being able to stimulate both types of genes, we find that the E1A regulatory protein enhances viral gene expression ~10 times more than β-globin gene expression. We also find that when connected to the *cis*-acting SV40 enhancer element, the β-globin gene cannot be further stimulated by the *trans*-acting E1A product. Finally, we find that transfection of either adenovirus or the β-globin gene into 293 cells, which constitutively expresses the E1A gene products, leads to an enhanced expression which is 10- to 20-fold higher than obtained by co-transfection of HeLa cells. The 293 cells thus provide a simple assay to demonstrate E1A-mediated transcriptional regulation.

Key words: 293 cells/E1A control of transcription/E4 expression/rabbit β-globin expression/transient expression

Introduction

Animal DNA viruses, like the adenoviruses, have contributed to our current understanding of eucaryotic gene expression. In particular, studies of the early region 1A (E1A, Figure 1) of subgroup C adenoviruses have provided insights into regulatory mechanisms which operate during virus replication. For example, region E1A has been shown to encode gene products which are required for both the activation of all the early viral transcription units (Berk *et al.*, 1979; Jones and Shenk, 1979a; Nevins, 1981; Riccardi *et al.*, 1981; Montell *et al.*, 1982) as well as being involved in the alteration of host cell metabolism during the infection (Nevins, 1981; Braithwaite *et al.*, 1983; Yoder *et al.*, 1983). Region E1A has also been shown to encode DNA sequences which are required for transformation of rodent cells by adenovirus (Graham *et al.*, 1978; Jones and Shenk, 1979b; Houwelling *et al.*, 1980) and recent studies have shown that region E1A enables the celular oncogene, T24 Harvey *ras* 1, to transform primary rat cells *in vitro* (Ruley, 1983).

The E1A transcription unit encodes three mRNAs of sizes 9S, 12S and 13S which are completely overlapping, i.e., they differ from each other by the size of the intron removed by RNA splicing (Figure 1C) (Berk and Sharp, 1977; Chow *et al.*, 1979; Spector *et al.*, 1978; Perricaudet *et al.*, 1979; Virtanen and Pettersson, 1983). Due to their overlapping nature the E1A 12S and 13S mRNAs are translated into two closely related proteins of 243 and 289 amino acids respectively; the difference being the deletion of 46 internal amino acids from the shorter protein product (Perricaudet *et al.*, 1979). Studies using adenovirus mutants having single base pair changes at strategic positions in region E1A have indicated that the E1A 13S mRNA product is responsible for the regulation of early viral gene expression (Riccardi *et al.* 1981; Montell *et al.*, 1982).

In contrast to all the other early viral genes, mRNA from the E1A transcription unit can also be efficiently expressed by plasmid transfection in HeLa cells (Svensson *et al.*, 1983; Weeks and Jones, 1983). This is probably because the upstream promoter region of E1A contains an enhancer sequence (Weeks and Jones, 1983; Hearing and Shenk, 1983; Imperiale *et al.*, 1983) which is efficiently recognized by the transcription factors present in the cell. Recent experiments have shown that the E1A-mediated regulation of early viral gene expression can be reproduced by plasmid co-transfection in tissue culture cells (Weeks and Jones, 1983; Elkaim *et al.*, 1983; Bos and ten Wolde-Kraamwinkel, 1983; Imperiale *et al.*, 1983). We have described the construction of recombinant plasmids which specifically express the E1A 13S and 12S mRNAs respectively (Svensson *et al.*, 1983). Here we present experiments where these plasmids have been used to demonstrate that the E1A 13S mRNA, or maybe more likely, the 289 amino acid protein product encoded by the E1A 13S mRNA, stimulates by a *trans*-acting mechanism the expression of genes of both viral and cellular origin. We show also that transfection into the 293 cell line, which constitutively expresses the E1A function (Aiello *et al.*, 1979) results in a very dramatic increase of specific gene expression.

Results

Enhanced expression of early region 4 mRNAs in cell co-transfected with E1A

Adenovirus early region 4 (Figure 1A) was selected for the initial studies of E1A-regulated gene expression since previous studies have shown that the E4 trancription unit is very sensitive to the action of the E1A gene products (Katze *et al.*, 1981; Weeks and Jones, 1983).

A recombinant plasmid encoding the E4 transcription unit was constructed by a two-step cloning procedure. First, *Eco*RI linkers were ligated to the molecular ends of the Ad2 genome. Following cleavage with endonuclease *Hind*III, the terminal fragments were inserted between the unique *Eco*RI and *Hind*III cleavage sites of plasmid pBR322. Clone pKGO-971 containing the right terminal *Hind*III-K fragment (map co-ordinates 97.2–100) was isolated by colony hybridization. To generate a plasmid containing the complete E4 trancription unit, fragment *Hind*III-F (map co-ordinates 89.5–97.2) encoding the body of the E4 regin was inserted in the correct orientation into plasmid pKGO-971, thus generating plasmid pKGO-895 (Figure 2A).

Plasmid DNA was introduced into subconfluent mono-

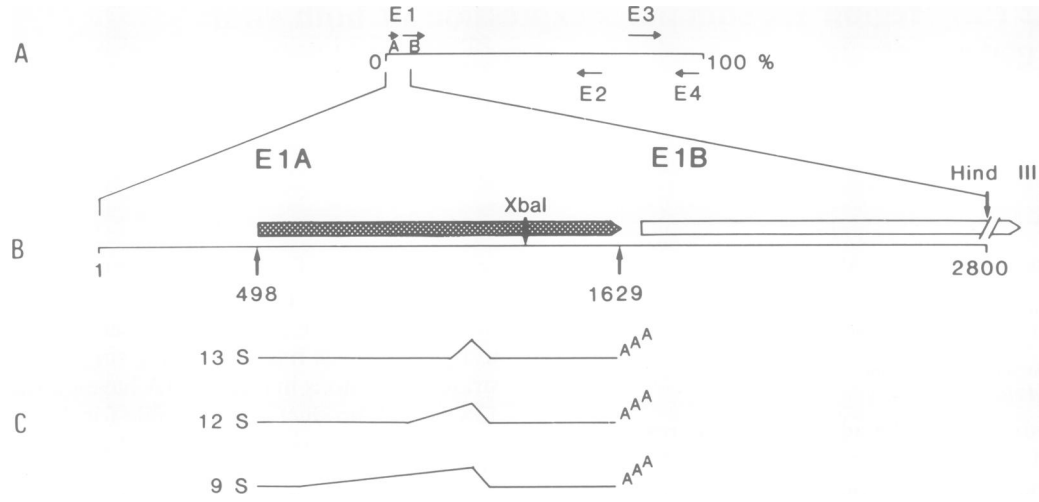

Fig. 1. Organization of the Ad2 genome. **(A)** A schematic drawing showing the location and direction of transcription of the five rgions expressed early after adenovirus infection. **(B)** Enlargement of a region located between map co-ordinates 0 and 7.7 in the Ad2 genome (fragment *Hind*III-G) encoding the E1A transcription unit and the 5′ portion of region E1B. **(C)** Structure of mRNAs encoded from region E1A.

layers of HeLa cells by the calcium phosphate co-precipitation technique (Wigler *et al.*, 1978). The transient expression of E4 mRNAs was measured by S1 endonuclease cleavage of cytoplasmic RNA isolated ~50 h post-transfection (Berk and Sharp, 1977; Weaver and Weissman, 1980). As a DNA probe a double-stranded DNA fragment 5′ end-labeled at the *Bgl*II cleavage site at map co-ordinate 95.7 was used (Figure 2B). From the known structure of the Ad2 E4 mRNAs it can be predicted that the acceptor site at map co-ordinate 96.6 (Chow *et al.*, 1979) will generate a S1 resistant fragment of 344 nucleotides (Virtanen *et al.*, in preparation).

The basal level of E4 mRNA expression in HeLa cells transfected with pKGO-895 is very low (Figure 2C). In contrast, co-transfection of pKGO-895 with increasing amounts of plasmid pKGO-007 SV-RI (Svensson *et al.*, 1983), which expresses all the E1A mRNAs efficiently, results in a drastic increase in E4 mRNA expression. A molar ratio of 1 between pKGO-895 and pKGO-007 SV-RI is sufficient to obtain maximum transcriptional stimulation. At optimal plasmid ratios a 50- to 100-fold stimulation of E4 mRNA expression was obtained by co-transfection with region E1A.

The potential to stimulate transcription is associated with the E1A 13S mRNA

Region E1A encodes three mRNAs, a 9S, 12S and 13S mRNA. We have previously described the construction of deletion mutants which precisely lack the 13S and 12S mRNA introns (pKGO-13S and pKGO-12S, Svensson *et al.*, 1983). These mutants, which have the altered capacity to encode either the E1A 13S or the E1A 12S mRNA products, were co-transfected in separate experiments with pKGO-895 DNA in order to identify the E1A gene product responsible for stimulating E4 mRNA expression. Plasmid pKGO-007 SV-RI, which contains the intact E1A region and pKGO-13S which encodes the E1A 13S mRNA both stimulate E4 mRNA expression ~100-fold (Figure 2C). In contrast, pKGO-12S which only expresses the E1A 12S mRNA was unable to stimulate E4 transcription. Furthermore, clone pJU 9S (Ulfendahl and Akusjärvi, unpublished), which expresses the E1A 9S mRNA and a severely truncated form of the E1A 13S mRNA was also unable to stimulate E4 mRNA expression (data not shown). Increasing the ratio of plasmid pKGO-12S relative to pKGO-895 in the transfection mixture, did not further increase E4 mRNA expression (data not shown), thus excluding the possibility that the inefficient expression of the E1A 12S mRNA in pKGO-12S transfected HeLa cells (Svensson *et al.*, 1983) accounts for the failure to stimulate E4 transcription.

Based on these results, we conclude that the E1A 13S mRNA, or maybe more likely, the 289 amino acid protein product encoded by the E1A 13S mRNA, is required for enhancement of E4 mRNA expression.

Stimulation of rabbit β-globin gene expression by E1A co-transfection

Both induction and shut-off of host cell gene expression occurs in adenovirus-infected cells (Beltz and Flint, 1979; Nevins, 1982; Yoder *et al.*, 1983; Braithwaite *et al.*, 1983). Since adenovirus gene expression is regulated at multiple levels by mechanisms that are still largely unknown, the interpretation of these results becomes complicated. To investigate the possible effect of the adenovirus E1A region on cellular gene expression, we studied the behaviour of a cloned rabbit β-globin gene (Maniatis *et al.*, 1978) in our co-transfection assay. Two recombinant plasmids were used (Banerji *et al.*, 1981), recombinant pSXβ$^+$ containing a 2.1-kb *Bgl*II fragment encoding the chromosomal rabbit β1-globin gene replacing the SV40 early region (Figure 3A); and recombinant pβ2x containing two tandem copies of a 4.7-kb *Kpn*I fragment encompassing the chromosomal rabbit β1-globin gene (Figure 4A). In agreement with earlier results (Banerji *et al.*, 1981) we find that globin mRNA expression is stimulated ~200-fold by the SV40 72-bp repeat (data not shown). By co-transfecting plasmid pβ2x and pSXβ$^+$ with plasmid pKGO-007 SV-RI into HeLa cells we determined the effect of the adenovirus E1A region on rabbit β-globin mRNA expression. As an internal control pKGO-895 DNA was included in the transfection mixtures to demonstrate that the recombinant plasmids were taken up and properly expressed in the transfected HeLa cells. Plasmid pSXβ$^+$ which efficiently expresses β-globin mRNA without the E1A function showed an unexpected slight decrease in β-globin expression after co-transfection with pKGO-007 SV-RI, pKGO-13S or pKGO-12S (Figure 3C). In general this decrease was between 2- and 3-fold (data not shown). In contrast, recombinant pβ2x lacking the SV40 enhancer element, showed an ~5-fold

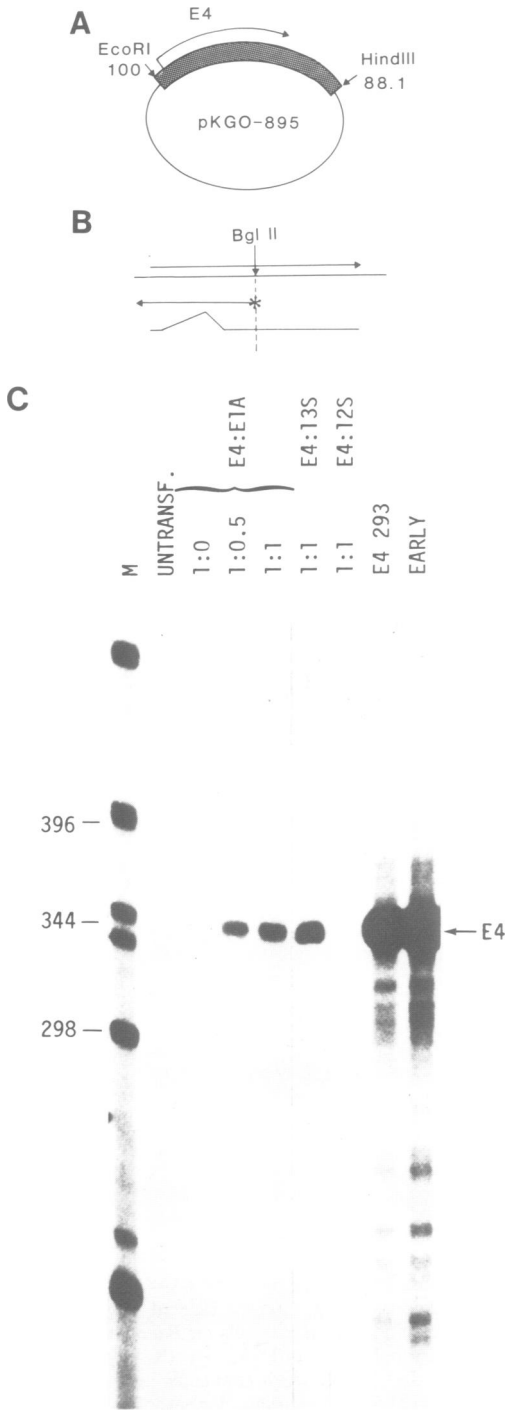

Fig. 2. Effect of early region 1A on the expression of early region 4 mRNA. **(A)** Schematic drawing illustrating the structure of recombinant plasmid pKGO-895. The stippled box indicates the 3.8-kb Ad2 DNA insert (map co-ordinate 88.1 – 100) with an arrow showing the direction of transcription for region E4. **(B)** Schematic diagram showing the position of the 5′ end-labeled *Bgl*II DNA fragment used for the S1 analysis of E4 mRNA. The 344 nucleoties long S1-resistant DNA fragment (panel **C**) corresponds to the distance from the *Bgl*II cleavage site (map co-ordinate 95.7) to the acceptor site for splicing at position 34733 (Virtanen *et al.*, in preparation). **(C)** S1 endonuclease analysis of E4 RNA accumulated in HeLa cells transfected with 6 μg of pKGO-895 DNA and various plasmids encoding region E1A. E4 represents pKGO-895 DNA; E1A, pKGO-007 SV-RI DNA; 13S, pKGO-13S DNA and 12S, pKGO-12S DNA. 1:0, no E1A plasmid added; 1:0.5, 3 μg of plasmid pKGO-007 SV-RI; 1:1, 6 μg of respective region E1A plasmid. E4 293, 6 μg of pKGO-895 DNA transfected into monolayers of 293 cells. Untransf., RNA isolated from untransfected HeLa cells. Early, RNA isolated from cells treated with AraC (25 μg/ml) from 1 to 7 h post-infection. M, pBR322 marker fragments.

stimulation of β-globin mRNA expression after co-transfection with pKGO-007 SV-RI or pKGO-13S (Figure 4B). As in the case of E4 mRNA expression, pKGO-12S co-transfection did not result in any stimulation.

Based on these results, we conclude that the potential of the E1A 13S mRNA product to stimulate expression is not restricted to adenovirus genes but includes also a cellular gene, the rabbit β-globin gene, when it is introduced into cells as an extra-chromosomal element. It should, however, be pointed out that expression of adenovirus region E4 is stimulated ~10 times more by region E1A than is the rabbit β-globin gene, suggesting a certain promoter specificity.

Enhanced expression of viral and cellular genes by transfection into 293 cells

Graham *et al.* (1977) have constructed a human embryonic kidney cell line transformed by fragments of adenoviurs type 5 DNA. These cells, designated 293 cells, express both mRNAs and viral proteins from early region 1 (Aiello *et al.*, 1979; Persson *et al.*, 1981). Since co-transfection of HeLa cells with plasmids encoding the E1A gene products results in a stimulation of both viral and non-viral transcription units, we tested the possibility that transfection of the 293 cell line would be a simple and efficient assay to investigate the action of the E1A gene products.

Transfection of plasmid pSXβ$^+$ containing the SV40 72-bp repeat into HeLa or 293 cells showed no stimulation of the rabbit β-globin mRNA expression (Figure 3D). This result was expected and confirms the conclusions drawn from our co-transfection experiments (Figure 3C). Since expression of the β-globin mRNA when under the control of the SV40 enhancer element does not respond positively to the action of the E1A products, pSXβ$^+$ DNA has whenever possible been included as an internal control for the transfection efficiency (all experiments except in Figure 4). Transfection of clone pKGO-895 into 293 cells resulted in an ~1000-fold enhancement of E4 mRNA expression as compared with HeLa cells (Figure 2C), although the level of β-globin mRNA synthesized from clone pSXβ$^+$ co-transfected into both cell types remained almost constant (Figure 3D), thus demonstrating the specificity of the E1A-stimulated expression of E4 mRNAs in 293 cells. Plasmid pβ2x showed a 50- to 100-fold stimulation in 293 cells (Figure 4C) corroborating our previous conclusions that adenovirus E1A stimulates the expression of the rabbit β-globin gene.

By transfection into 293 cells we reproducibly obtain a 10- to 20-fold better stimulation than by co-transfection of HeLa cells. The almost identical behaviour of plasmid pSXβ$^+$ in HeLa and 293 cells strongly suggests that the enhanced gene expression in 293 cells is caused by the E1A products present in the 293 cell, rather than some unspecific feature, such as differences in uptake of DNA.

Discussion

The 289 amino acid protein encoded by the E1A 13S mRNA controls early viral gene expression (Riccardi *et al.*, 1981; Montell *et al.*, 1982), by a mechanism which is still uncertain. By measuring the rate of transcription from the early viral promoters, Nevins (1981) postulated that the E1A gene product acts at the level of transcriptional initiation and recent experiments have confirmed this hypothesis (Weeks and Jones, 1983; Elkaim *et al.*, 1983). Since the largest E1A protein appears to activate transcription it is not surprising that the protein has been found in nuclei of infected cells

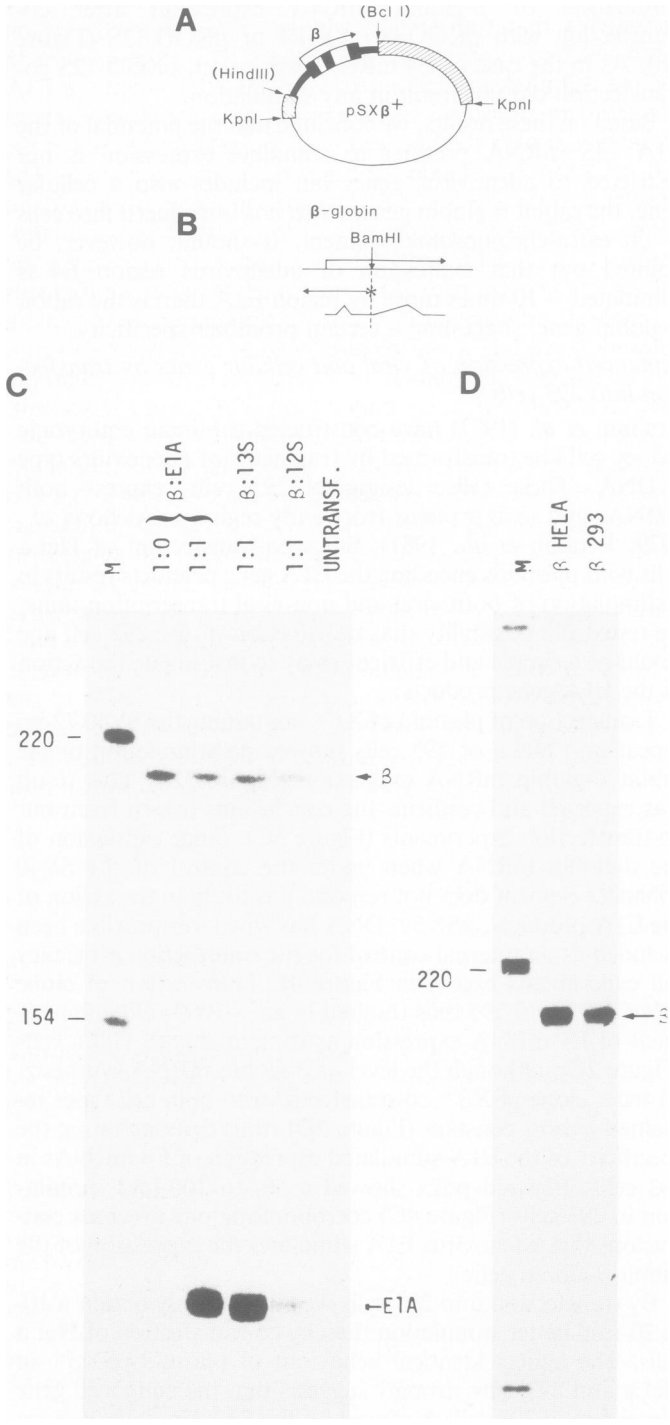

Fig. 3. Effect of adenovirus region E1A on rabbit β-globin gene expression from plasmid pSXβ+ containing the SV40 enhancer element. **(A)** A schematic representation of the recombinant plasmid pSXβ+ (for details see Banerji *et al.*, 1981) used in the experiment. Thin line, pBR322 sequences; hatched area, SV40 sequences; solid area, rabbit DNA; open bars denote the introns in the rabbit β-globin gene. **(B)** S1 nuclease mapping scheme. Rabbit DNA 5′ end-labeled at the *Bam*HI cleavage site (for further details see Banerji *et al.*, 1981) and pKGO-007 DNA 5′ end-labeled at the *Xba*I cleavage site unique in the E1A region (for further details see Svensson *et al.*, 1983) was mixed and hybridized to unlabeled RNA isolated from transfected cells and treated with nuclease S1. Resistant material was separated electrophoretically in a denaturing polyacrylamide gel. **(C)** S1 endonuclease analysis of the β-globin and E1A RNAs accumulated in HeLa cells transfected with 6 μg pSXβ+ DNA and various plasmids encoding region E1A. Symbols are as described in the legend to Figure 2C. **(D)** S1 nuclease analysis of rabbit β-globin mRNA expression in HeLa and 293 cells transfected with 6 μg of pSXβ+ DNA. Symbols are as in Figure 2C.

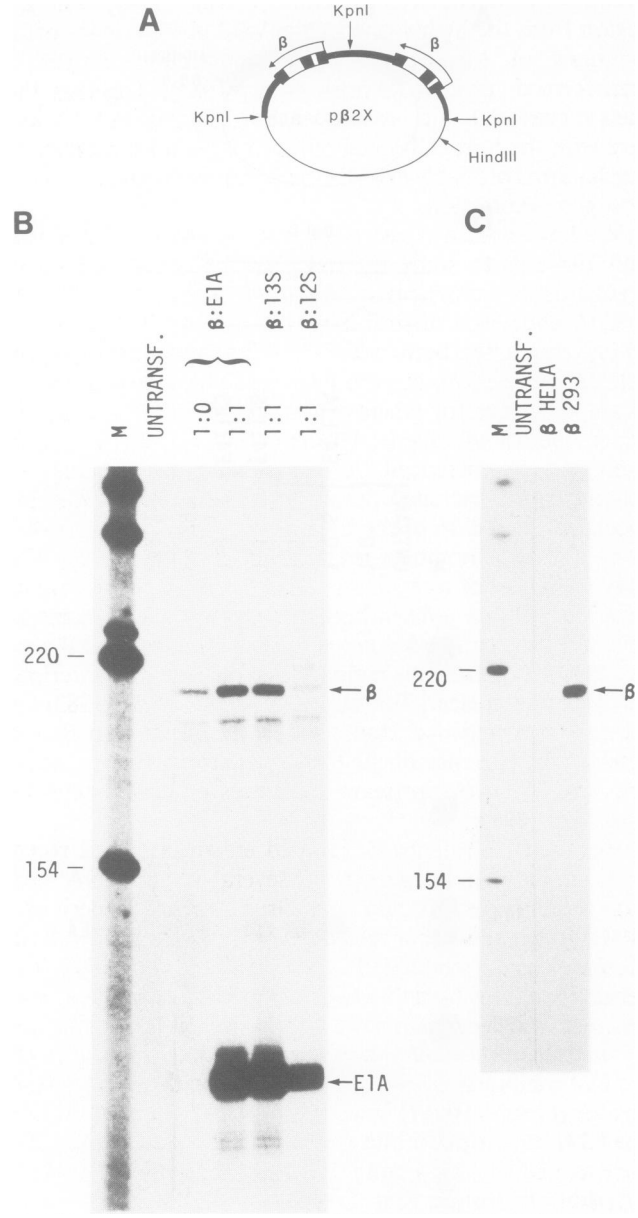

Fig. 4. Effect of adenovirus E1A on rabbit β-globin gene expression from plasmid pβ2x lacking the SV40 enhancer element. **(A)** A schematic diagram of recombinant plasmid pβ2x (for details see Banerji *et al.*, 1981) used in the experiment. Symbols are as in Figure 3A. **(B)** S1 endonuclease analysis of rabbit β-globin and E1A RNAs accumulated in HeLa cells transfected with 6 μg of plasmid pβ2x and various clones encoding region E1A. The strategy for S1 analysis was as described in Figure 3B. Symbols are as described in the legend to Figure 2C. **(C)** S1 nuclease analysis of β-globin RNA accumulated in HeLa and 293 cells transfected with 6 μg of plasmid pβ2x. Symbols are as in Figure 2C.

(Feldman and Nevins, 1983; Yee *et al.*, 1983; Spindler *et al.*, 1983). A post-transcriptional control of early viral gene expression at the level of mRNA accumulation has also been identified (Katze *et al.*, 1981), thus suggesting that the E1A gene products may control multiple levels of adenovirus gene expression.

Region E1A also causes cell cycle abnormalities during lytic infection. For example, it has been implicated in the induction of thymidine kinase synthesis and cellular DNA replication (Braithwaite *et al.*, 1983) and also in the induction of a HeLa cell 70 000 dalton heat-shock protein during the early

phase of an adenovirus infection (Nevins, 1981). The E1A region from the highly oncogenic Ad12 also switches off the synthesis of the class I histocompatibility antigens in transformed rat cells (Schrier *et al.*, 1983). Together these data suggest that the proteins encoded by region E1A interfere with the host cell metabolism in a complex manner and mechanisms probably exist for both induction and shut-off of host gene expression.

We have used a transient expression assay in both HeLa and 293 cells to study the specificity of the E1A-mediated regulation of transcription. We show here that the E1A 13S mRNA encodes a diffusible product, most likely a protein, which stimulates both adenovirus E4 and rabbit β-globin mRNA production, but the E1A regulatory protein shows a clear preference for adenovirus genes. The DNA sequences which interact with the E1A 13S mRNA product have not yet been fully characterized. A region between −94 and −63 relative to the cap site appears to be required for the E1A-mediated regulation of the EIIa early gene promoter (Elkaim *et al.*, 1983). A homologous decanucleotide sequence, which may form part of a regulatory signal, has also been found at position −70 to −80 in both the rabbit β-globin gene and several of the early adenovirus transcription units (Elkaim *et al.*, 1983). Whether this region plays any role in transcription regulation is unclear. For example, Green *et al.* (1983) have recently reported that transcription of the human β-globin gene, in the presence of the E1A regulatory protein, requires only the 36 bp of upstream sequences covering the TATA box.

Regulatory elements, designated enhancers, have recently been identified in the genome of several animal DNA viruses (for a review see Khoury and Gruss, 1982). Banerji *et al.* (1981) have shown that mRNA expression from the rabbit β-globin gene is stimulated ~200-fold by the SV40 72-bp repeat. We show here that β-globin mRNA expression from a plasmid, which carries the SV40 enhancer, is insensitive to the E1A-mediated stimulation of transcription. Instead we detect a slight inhibition of β-globin mRNA expession by plasmids encoding region E1A (Figure 3C). Since all plasmids encoding the E1A transcription unit (pKGO-007 SV-RI, pKGO-13S as well as pKGO-12S; Figure 3C) reduce β-globin mRNA expression, we suspect that the presence of the E1A structural gene, rather than any of the E1A polypeptides, causes the reduction in β-globin mRNA synthesis. A simple explanation for the observed result would be that the factors required for transcription of exogenously added DNA are available in limited amounts in the transfected cells. Thus, the slight decrease in β-globin mRNA expression in cells co-transfected with region E1A may be the result of a competition between the two transcription units for factors required for transcription.

We have found that genes that are stimulated by E1A co-transfection are also stimulated when transfected into 293 cells (Graham *et al.*, 1977), which constitutively express the mRNAs as well as the proteins from region E1 (Aiello *et al.*, 1979). The stimulation in 293 cells is reproducibly 10−20 times better than after E1A co-transfection. Plasmid pSXβ$^+$, which is almost unaffected by E1A co-transfection, shows no enhanced expression after transfection into 293 cells either (Figure 3C and 3D), thus suggesting that the transcriptional stimulation observed is specific and most likely is dependent on the E1A proteins produced in the 293 cells.

Comparable amounts of E1A mRNAs are produced in transfected HeLa and 293 cells (data not shown) suggesting that equal amounts of E1A proteins are produced. The possibility that transfection with plasmids encoding region E1A results in lower amounts of the E1A regulatory protein in the cell nucleus, compared with 293 cells where the E1A proteins are constitutively expressed, and therefore exists in equilibrium with the host cell, may provide an answer to the 10- to 20-fold difference in transcriptional stimulation. The presence in the 293 cell line of a functional E1B region (Aiello *et al.*, 1979) may of course be of importance, since a combination of the E1A and E1B gene products may enhance the effect of the E1A regulatory protein. However, we find this hypothesis unlikely since co-transfection experiments using a plasmid encoding a complete E1 region does not result in a more efficient stimulation of transcripton than is observed with a plasmid encoding only region E1A (data not shown). Although the co-transfection experiments have been performed with plasmids encoding the Ad2 E1A and the 293 cell line express the Ad5 E1A region, we do not believe that this difference provides the explanation for the 10- to 20-fold difference in transcriptional stimulation, since both adenovirus type 2 and 5 belong to the non-oncogenic subgroup C adenoviruses and show a homology which exceeds 95% (Mackey *et al.*, 1979). To distinguish between these alternatives we have started experiments to quantitate the amount of E1A proteins synthesized, and to determine the cellular localization of the E1A proteins in transfected cells. We cannot yet rule out the possibility that a property unrelated to the adenovirus E1A gene function is responsible for the increased stimulation of gene expression in 293 cells.

Materials and methods

DNA, RNA and enzymes

Cloning in plasmid vectors including the addition of synthetic *Eco*RI linkers (dGGAATTC, Bethesda Research Labortories) were according to standard recombinant DNA techniques (Maniatis *et al.*, 1982) using pBR322 as a vector molecule. Isolation of cytoplasmic RNA from HeLa monolayer cells and 293 cells transfected with recombinant plamids was as described previously (Akusjärvi and Pettersson, 1978). All enzymes except nucleases S1 (Sigma) and polynucleotide kinase (New England Nuclear) were purchased from New England BioLabs.

Cell growth and transfection

Monolayer cultures of HeLa and 293 cells were maintained in Dulbecco's modified Eagle's medium with 10% (v/v) calf serum or fetal calf serum respectively, and 50 μg gentamicin/ml. Transfection of subconfluent cells was performed as previously described (Svensson *et al.*, 1983) using the calcium phosphate co-precipitation technique (Wigler *et al.*, 1978). Usually, a total of 15 μg of plasmid DNA were transfected per 6 cm Petri dish. In co-transfection experiments, plasmids were added in the amount stated in the figure legends. Whenever possible, 3 μg of pSXβ$^+$ was added as an internal control of the transfection efficiency. When necessary, pBR322 DNA was added to give a final amount of 15 μg of plasmid DNA per transfection.

S1 endonuclease analysis

The protocol described by Svensson *et al.* (1983) was followed with a few minor modifications. Usually 10 μg of total cytoplasmic RNA isolated from transfected cells (0.1−1 μg of RNA from early Ad2-infected cells) was hybridized overnight at 56°C (β-globin and E1A) or 47°C (E4) to the 5′ end-labeled DNA fragments indicated in the figure legends. S1 nuclease cleavage and electrophoretic separation was on 4% (Figure 2) or 8% (Figures 3 and 4) polyacrylamide gels containing 8 M urea as previously described (Svensson *et al.*, 1983).

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