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Activation of Epstein-Barr virus promoters by a growth-factor and a glucocorticoid

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Transforming growth factor-β (TGF-β) and a glucocorticosteroid, Dexamethasone (DXM), both cause transcriptional induction of Epstein-Barr virus (EBV) early antigens (EA) in Daudi lymphoma cells. The viral induction occurs through the viral promoter DR overlapping an origin of replication which is active during the lytic cycle. Each hormone requires specific regions on the DR promoter. Since these regions also mediate the action of two viral transcription factors, EB1 and R, it may be emphasized that EB1 and/or R are involved in the EA induction process by TGF-β and by DXM.

Transforming growth factor-β; Dexamethasone; Transcription; Early antigen; Epstein-Barr virus; Lymphoma cell

1. INTRODUCTION

The transforming growth factor-β (TGF-β) belongs to a subgroup of a large family of peptidic hormones [1] and is known to display a variety of regulatory functions in normal and in neoplastic cells. TGF-β affects cell proliferation and differentiation by exerting either a stimulatory or an inhibitory effect. Of special importance is the suppressive action of TGF-β on the growth of T and B lymphocytes, suggesting practical applications as an anti-inflammatory or an immunosuppressive agent [2]. It has recently become clear that, in addition to its role in growth control, TGF-β stimulates the synthesis of several extracellular matrix proteins, including type I collagen and fibronectin [3], and inhibits the expression of several proteases [4]. The latter activities confer to TGF-β a major physiological role in wound healing [5].

It is well established that the action of TGF-β is mediated by an interaction between the growth factor and a specific receptor located in the plasma membrane. It is clear that TGF-β acts on gene expression primarily at the transcriptional level [6–9].

Abbreviations: CAT, chloramphenicol acetyl transferase; DR, duplicate right; DXM, dexamethasone (9α-fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregna-diene-3,20-dione); EA, early antigens; EBV, Epstein-Barr virus; NF1, nuclear factor 1; TGF-β, transforming growth factor-β; TPA, 12-*O*-tetradecanoyl-phorbol 13-acetate; RU486, 17β-hydroxy-11β-(4-dimethylaminophenyl)-17α-(1-propynyl)-estra-4,9-diene-3-one.

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Much information is available on the regulation of transcription of cellular and viral genes mediated by glucocorticoid hormones (for a review see [10]). In B cells latently infected with the Epstein-Barr virus (EBV), a double-stranded DNA Herpes virus, TGF-β, and the potent synthetic glucocorticoid, Dexamethasone (DXM), are both able to induce the synthesis of viral early antigens (EA) [11]. Therefore, we decided to compare the effects of TGF-β and DXM at the transcriptional level. Using the CAT system developed by Gorman et al. [12] we analysed the target sequences involved in the induction of the EBV early promoters.

2. MATERIALS AND METHODS

2.1. *Steroids*

DXM (9α-fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregna-diene-3,20-dione) and RU486 (17β-hydroxy-11β-(4-dimethylamino-phenyl)-17α-(1-propynyl)-estra-4,9-diene-3-one) were kind gifts from Roussel UCLAF. TGF-β was either prepared by D.A. Lawrence [13] or purchased from R&D (Minneapolis, USA).

2.2. *Cell lines and steroid treatments*

EBV-positive Daudi cells were grown in RPMI 1640 supplemented with 10% charcoal-treated foetal calf serum and 2 mM glutamine in a 5% CO_2 atmosphere at 37°C. For induction, TGF-β (5 ng/ml) and steroids (3 μM) were added to lymphoid cells during 48 h.

2.3. *DNA templates*

Plasmidic construction pKCAT was obtained by inserting the EBV promoter DR in front of the bacterial CAT gene coding sequence [14]. Deletion constructs were obtained as described previously [15]. Plasmid pNCAT, which contains 2 kb of the promoter controlling the expression of the EBV transcription factor EB1 in front of the CAT reporter gene, was a gift from Urier G. (unpublished results).

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2.4. *Transfection experiments*

One day before transfection, Daudi cells were suspended at a 5×10^5 cell density per ml in fresh medium. Lymphoma cells were transfected with 2 μg of CAT reporter gene by the DEAE-dextran method [16]. Before induction, the transfected cells were pooled and sampled in order to get the same transfection efficiency in each assay.

2.5. *CAT assays*

These tests were essentially performed as described earlier [12]. The sonication was replaced by lysing, the cells during 5 cycles of freezing-thawing. After centrifugation, the supernatant was assayed for CAT activity. The reaction time and quantity of extract were selected to stay in the linear range of the reaction. Protein concentration was determined with the BioRad assay kit.

3. RESULTS

3.1. *TGF-β and DXM activate the DR promoter*

Daudi lymphoma cells were transfected with the recombinant plasmid pKCAT, which contains, inserted 5′ to the bacterial CAT encoding gene, the EBV early promoter DR. This promoter controls the EA synthesis and overlaps an origin of replication active only during the lytic cycle [17] (Fig. 1). Transfected cells were grown either with or without TGF-β (5 ng/ml) or DXM (3 μM) for 48 h, and CAT expression was measured.

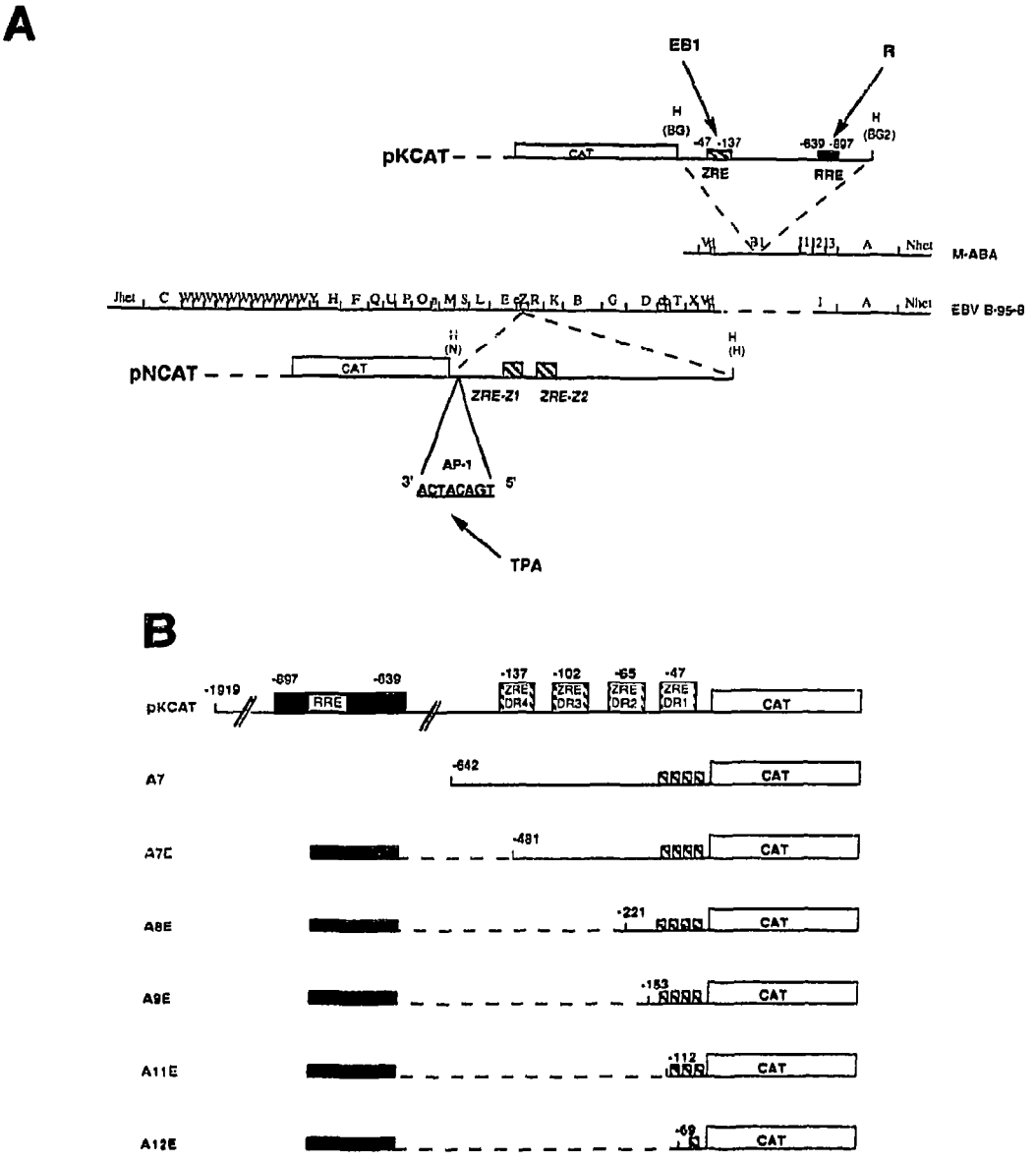

Fig. 1. (A) Linear representation of the EBV genome and of the plasmids pKCAT and pNCAT. pKCAT contains the CAT coding sequence under the control of the EBV DR promoter. The localisations of the sequences responsive to the viral transcription factors EB1 and R are indicated respectively as ZRE and RRE elements. pNCAT contains the promoter of EB1 in front of the CAT coding sequence; the localisations of the AP1 sequence and of the EB1 responsive sequences, ZRE-Z1 and ZRE-Z2 are indicated. (B) Deletion mutants of the DR promoter in pKCAT construct. The black box represents the distal enhancer element containing the R responsive element, e, and the hatched boxes represent the EB1 binding sites. Restriction sites abbreviations: H = *Hin*dIII; N = *Nae*I; BG = *Bgl*I; BG2 = *Bgl*II.

The addition of TGF-β to Daudi cells caused a marked increase in the level of CAT activity (Fig. 2). Maximal stimulation of CAT activity was measured with a 5 ng/ml concentration of TGF-β (data not shown). This value is close to the amount required for TGF-β to express other effects like autoactivation of its own promoter or activation of the collagen promoter [18]. By comparing the induction due to TGF-β or to DXM, a higher level of CAT induction was observed with the growth factor. Routinely, a 17-fold induction and a 9-fold one were measured respectively with TGF-β (lane 1) and the glucocorticoid (lane 2). When the two compounds were given simultaneously, an additive induction ratio of about 23-fold was observed (lane 4). The CAT induction observed with TGF-β or DXM represents a true activation of the DR promoter. It is not a consequence of cell proliferation, since no change in the growth rate occurred in the presence of the drugs.

The induction of CAT expression suggests that both TGF-β and DXM act directly or indirectly on the DR promoter. Moreover, the two compounds did not interfere with one another since simultaneous administration leads to an additive effect. This observation also suggests that the two inducers do not act through the same target(s) on the DR promoter.

3.2. *Effects of TGF-β and DXM on mutants of the DR promoter*

In order to determine which sequences are involved in the induction of the DR promoter by TGF-β and glucocorticoids, we tested several deletion mutants (Fig. 1B). Deletion of the region between −897 and −639 (mutant A7) reduced TGF-β induction to the basal level (Fig. 3A). In order to identify other regulatory elements in this promoter, region −897 to −639 was positioned at an approximate constant distance from the CAP site as it occurs in the wild-type promoter (mutants A7E to A12E, Fig. 1B). CAT expression was not decreased by more than 50% of the maximal induced level (referring to the pKCAT construct), providing the deletion was over position −112. Deletions under position −112 until −69 bp led to a drastic decrease to the basal level. These data suggest that the sequences mediating the response to TGF-β are located in two distinct regions: one is located between

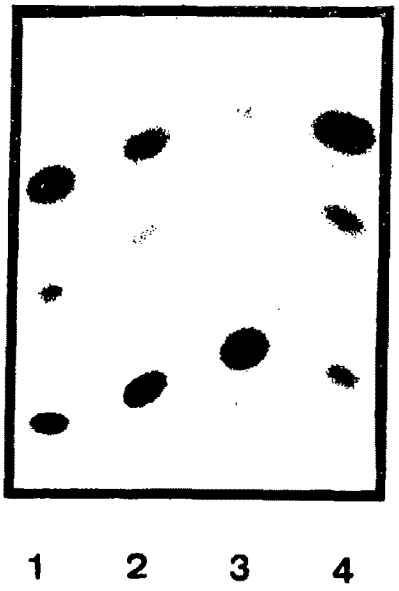

Fig. 2. Effect of DXM and TGF-β on the DR promoter in Daudi cells. CAT assays were performed essentially as described in section 2. Extract from cells transfected with pKCAT (lane 1) in presence of TGF-β (5 ng/ml); (lane 2) in the presence of DXM (3 μM); (lane 3) in the absence of inducers; (lane 4) in the presence of TGF-β (5 ng/ml) and DXM (3 μM). The data presented correspond to a typical experiment.

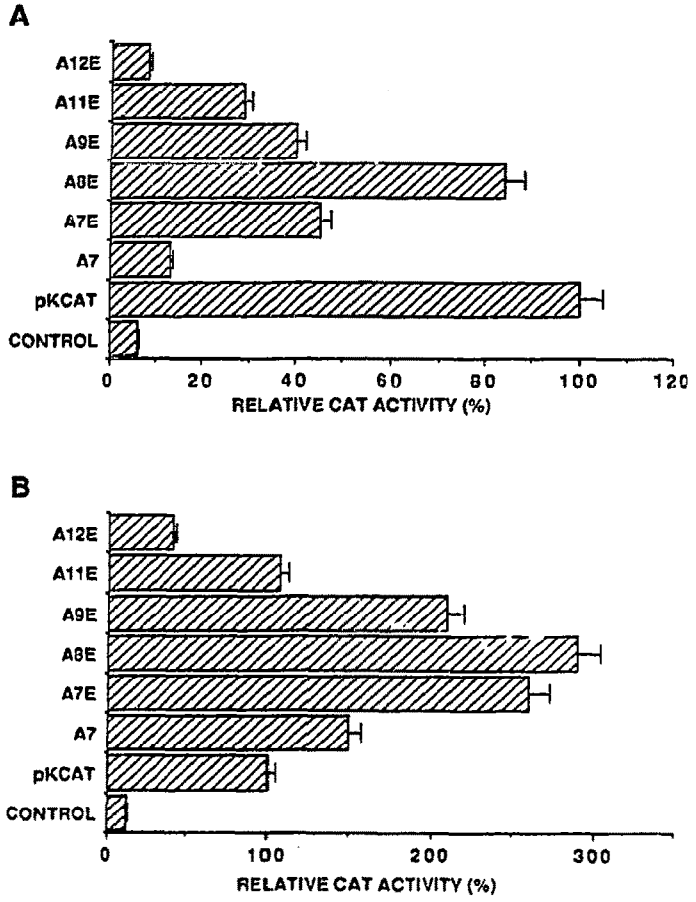

Fig. 3. Effect of DNA deletions in the DR promoter on CAT expression induced by TGF-β or DXM. Daudi cells were transfected with pKCAT deletion mutants and treated with TGF-β (5 ng/ml) (A) or DXM (3 μM) (B) during 48 h. Percent of CAT induction is determined by comparison with the CAT induction obtained with the original pKCAT construct in the presence of TGF-β or DXM. Control cultures transfected with pKCAT were untreated. CAT activities are the mean of two duplicate independent experiments. 100% represents 0.5 μmol of chloramphenicol acetylated per min and per mg of protein for TGF-β induction and 0.25 μmol of chloramphenicol acetylated per min and per mg of protein for DXM induction. Endogenous steroids have been discarded from the medium. Bars = SD.

nucleotide −897 and −639 and the other one between nucleotide −112 and −69.

The same series of mutants were used to analyse the transient expression under DXM treatment (Fig. 3B). We observed that the −897 to −639 region was not a prerequisite for DXM inducibility. Moreover, the stimulatory effect of DXM was higher with the mutants than with the wild-type construct. Indeed, the CAT activity obtained after deletion of the region spanning nucleotides −642 to −481 was 3-fold above the induction rate measured with pKCAT. This observation suggests an inhibitory role of this region in the induction process by DXM. However, when the deletion was extended beyond position −112, CAT expression drastically decreased, as observed with TGF-β. These experiments suggest that only one region located between −112 and −69 is required to mediate DXM activation of the DR promoter.

From these results, we may conclude that the sequence between −112 and −69, proximal to the TATA box, contains an important regulatory sequence which plays a role for the responsiveness of the DR promoter to TGF-β and to glucocorticoids. In addition, TGF-β requires the presence of a distal sequence, spanning nucleotide −897 to −639, to express its inducibility through the DR promoter.

3.3. *Effect of TGF-β on the EB1 promoter*

Recently it has been reported that four binding sites for the viral transcription factor EB1 were located in the region spanning bp −69 to −112 [19], whereas an R-responsive enhancer is found between bp −639 and −847 [20,21]. Since EB1 activates its own promoter [22,23] and the R promoter [24], we tested the effect of TGF-β on the EB1 promoter. Cells were transfected with plasmid pNCAT which contains about 2 kb of the EB1 promoter region in front of the CAT gene (Fig. 1A). Neither TGF-β nor DXM significantly induced CAT expression through the EB1 promoter in Daudi cells, suggesting that the two hormones do not up-regulate the expression of transactivator EB1 (data not shown). Therefore, the effects of TGF-β and DXM are likely to be mediated by DR promoter sequences, other than EB1 and R responsive elements. Nevertheless, in the case of TGF-β induction, a potentiation by EB1 and R may occur.

3.4. *Antagonistic action of RU486 on TGF-β or DXM induced CAT expression*

The synthetic steroid, RU486, which is acting as a potent antiglucocorticoid by inhibiting the synthesis of the viral EA induced by DXM, was also shown to slow down the EA induction brought over by TGF-β [11]. Our previous work led us to investigate how the steroid antagonist interferes on the DR promoter-dependent induction of CAT expression. Therefore, Daudi cells were transfected with pKCAT and submitted to 48 h

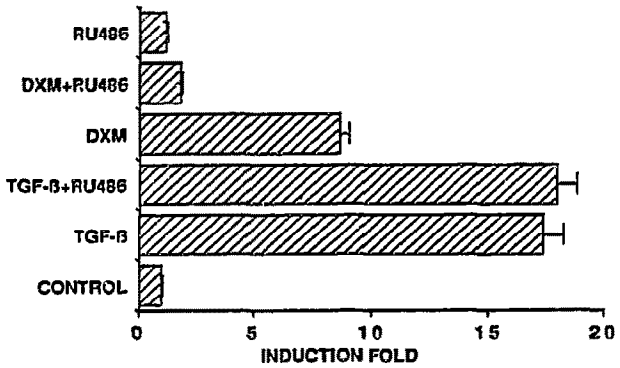

Fig. 4. Daudi cells were transfected with pKCAT and treated with TGF-β (5 ng/ml) or DXM (3 μM) alone, or in combination with RU486 (3 μM). CAT activity was determined 48 h after transfection. Induction-fold is determined in comparison with CAT activity obtained with untreated Daudi cells transfected with pKCAT. The results are the mean of 3 duplicate independent experiments. Endogenous steroids have been discarded from the medium. Bars = SD.

treatments with the different combinations of DXM, TGF-β and RU486 as shown in Fig. 4. RU486 did not modify the TGF-β-inducing activity on the DR promoter. These results do not correlate with the inhibitory effect exerted by RU486 on the EA expression measured in the cells by immunofluorescence [11]. More work is required to understand this discrepancy which may depend on an interaction of RU486 at the post-transcriptional level.

In contrast, RU486 exerts a strong antagonism of DXM induction at the transcriptional level; this result strengthens the fact that the CAT induction by DXM reflects a specific hormonal effect.

4. DISCUSSION

The transient expression experiments reported here demonstrate that the treatment of Daudi cells with TGF-β or DXM causes an activation of the viral DR promoter. This transcriptional activation could account to a large part for the increased EA synthesis observed earlier by immunofluorescence experiments [11]. During this study, we localised specific regions in the DR promoter mediating the effect of TGF-β and of DXM. Two regions are involved in the activation by TGF-β. The first one, located close to the TATA box (−112 to −69 bp), is known to be also responsive to the EBV transactivator EB1 [16,19]. This viral transcription factor is involved, with the EBV enhancer factor R, in the disruption of viral latency by activating the EBV early promoters. The second region spanning bp −897 to −639 is an enhancer inducible by the EBV-early-factor R [24]. Thus two viral proteins, EB1 and R, might be directly or indirectly involved in the EA induction process by TGF-β. Since TGF-β does not activate the expression of EB1, an indirect process might be emphasized.

Recently, several authors have described the interaction of cellular proteins as second messengers for TGF-β induction. The activation of the collagen promoter by TGF-β was shown to be mediated by a specific sequence corresponding to a binding site for nuclear factor 1 (NF1) [6]. The autoactivation of the second promoter of the TGF-β1 gene was shown to be mediated by the AP-1 (jun/fos) factor. Therefore there exist common targets for 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA) and TGF-β on the DNA [7,8,25]. Conversely, gene inhibition by TGF-β involves binding of a Fos-containing protein complex to a promoter sequence in the transin/stromelysin gene promoter termed the TGF-β1 inhibitory element [26]. A computer search on the DR promoter did not reveal any NF1-specific sequence in this promoter. In contrast, there is a TPA-responsive element in the EB1 promoter which is known to be responsive to TPA [24,25]. However, no induction of the EB1 promoter has been observed under TGF-β treatment.

This present study suggests that the transcriptional regulation of EBV genes by TGF-β is quite complex. Multiple regulatory mechanisms inducing the synthesis or the activation of various factors, such as EB1 and R, seem to be intimately involved in eliciting EA synthesis. Presently the responsiveness to TGF-β and DXM is not solved. This preliminary study prompted us to additional experiments including DNA footprinting and gel shift assays in order to draw a clear relationship between the induction process and specific transcriptional factors which have to be identified.

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