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Role of a GC-rich motif in transcription regulation of the adenovirus type 2 IVa2 promoter which lacks typical TATA-box element

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Promoter of the adenovirus type 2 IVa2 (IVa2) gene does not have a TATA-box element, hence it is considered to be a model of cellular TATA-less promoters. In vitro transcription of the IVa2 promoter in HeLa cell extracts identified a proximal *cis*-element around −40 relative to the transcription start site. DNase I footprint analysis revealed the presence of a DNA-binding factor which interacts with sequences ACCCCTCCCACTTAG at −50. Competition footprint analysis and sequence comparisons showed that the IVa2 proximal promoter element is equivalent to the GC-box, the Sp1-binding site.

Adenovirus type 2 IVa2 promoter; Transcription in vitro; Transcriptional regulation; Sp1; DNase I footprint

1. INTRODUCTION

RNA polymerase II-dependent genes contain three types of *cis*-elements for efficient and tissue-specific transcription, which include the TATA-box, upstream element and enhancer. The TATA-box which is located some 30 base pairs (bp) upstream from the transcription start site [1], is a target site of TFIID; one of the general transcription factors. TFIID binds to the TATA-box and conducts formation of the preinitiation complex [2,3]. There are another class of promoters which lack TATA-box. These genes often harbor GC-rich sequence motifs in their proximal promoters. However, mechanisms of transcription initiation of TATA-less promoters are poorly understood. The adenovirus type 2 IVa2 gene (IVa2) which is expressed at an intermediate time during the viral infection, lies at 211 bp upstream from the major late promoter (MLP) [4]. Since the IVa2 gene does not have a typical TATA-box, this promoter has been a good model of cellular TATA-less promoters. Natarajan et al. [5,6] have demonstrated that the IVa2 promoter contains at least one proximal and one complex distal *cis*-element at −38 and −152, respectively. The distal element overlaps with a control region of the MLP. In the distal promoter, one positive *trans*-acting factor was shown to bind to sequences between −152 and −179 [7]. However, little has been known about the proximal promoter. Here, we demonstrate by in vitro analyses that a GC-rich motif in the IVa2 proximal promoter is equivalent to the Sp1-binding and involved in efficient transcription.

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Abbreviations: IVa2, adenovirus type 2 IVa2 gene; MLP, adenovirus type 2 major late promoter; bp, base pairs; PPE, proximal promoter element; DHFR, dihydrofolate reductase

2. MATERIALS AND METHODS

2.1. *Plasmids and DNA construction*

pIV86 DNA carries the IVa2 sequences from −86 (*Sma*I) to +468 (*Eco*RI), and the number of other pIV-series DNA represents the 5′-terminus of the IVa2 sequence. A *Bam*HI linker was put to the 5′-terminus of pIV-series DNAs, and the resultant *Bam*HI-*Eco*RI fragment was cloned into pBR322. The pIV-series deletion mutants were made from pIV86 DNA by *Bal*31-digestion. DNA construction and plasmid preparation were done according to the standard method [8].

2.2. *Extract and in vitro transcription*

HeLa cells extracts (6 mg/ml) were prepared according to the standard method [9]. In vitro transcription was performed as previously described [10]. Each reaction (20 μl) contains 150 ng of one of the *Eco*RI-cut pIV-series DNAs (experimental) and 150 ng of *Nco*I-cut pIV86 DNA (reference). Reaction was terminated after incubation at 25°C for 45 min, and run-off transcripts were analyzed through 5% sequencing gels.

2.3. *DNase I footprint*

The standard DNase I footprint assay consists of a 20 μl reaction mixture as previously described [11]. The reaction mixture containing 0–4 μl of HeLa cell extracts and non-specific pBR322 DNA (200 ng) was incubated for 10 min on ice (preincubation). Two nanograms of ^{32}P-labeled DNA probe (10 kcpm) was added to the mixture and incubated at 25°C for 10 min. DNAs were digested with DNase I (80 ng) in the presence of 3 mM $CaCl_2$ at 25°C for 1–3 min. The digests were analyzed through 8% sequencing gels. Competition footprint assay was done as described above except that competitor DNA was added at the preincubation period.

3. RESULTS

3.1. *Transcription of the adenovirus IVa2 promoter in HeLa cell extract*

The IVa2 promoter is located 346 bp upstream from the MLP [4] (Fig. 1). We analyzed the promoter strength of the IVa2 gene in HeLa cell extracts. pIV86 carrying IVa2 sequences from −86 to +468 relative to the transcription start site was used as a parental DNA for further 5′-deletion mutants. The deletion mutants (pIV-series DNAs) were constructed by *Bal*31 nuclease digestion. DNAs were cut with *Eco*RI and run-off transcription assay was performed (Fig. 2). The *Nco*I-cut pIV86 was mixed with each reaction to standardize reactions. We found that pIV64 and pIV50 were transcribed as well as pIV86. However, pIV37 DNA exhibited a considerably weak promoter activity (20% of pIV86) (Fig. 2, lane 4). The rest of the mutants carrying a larger deletion had lower transcription activity than pIV37, and transcription of pIV9 was very faint. These results demonstrated that there was a potent *cis*-acting promoter element downstream from −50.

3.2. *Binding factor of the IVa2 proximal promoter element*

The fact that mutation in a particular promoter sequence causes a loss of transcription activity generally suggests an interaction of factors with specific DNA sequences. We carried out a DNase I footprint experiment using HeLa cell extracts (Fig. 3A). To analyze footprint patterns especially around −50, IVa2 DNA was labeled with ^{32}P at −159 for the non-coding strand probe or at +51 for the coding strand probe, and subjected to DNase I digestion. As for the non-coding strand, DNA stretch from −36 to −50 was strongly protected against DNase I digestion in the presence of extract. Another partial protection was observed between −53 and −79. A DNase I-hyper-digested band was observed at −32. As for the coding strand, strongly protected and hyper-digested regions were seen at −38 to −48 and −52 to −57, respectively. Weak protections were observed at −61 to −77. These results demonstrated that sequences around −40 bind strongly to a cellular factor. Another binding site was observed around −70. We schematically illustrated the strong footprint region in Fig. 3B. Interestingly, we found that the major protected sequences overlapped with the *cis*-element described above. Hence, we suggest that effi-

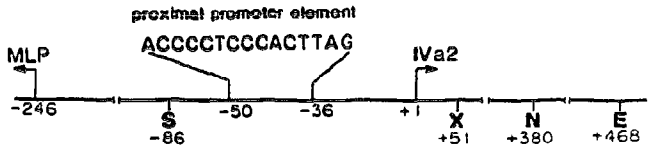

Fig. 1. Structure of adenovirus type 2 DNA around the IVa2 promoter. Nucleotide sequence (upper strand) of the proximal promoter element (PPE) between −50 and −36 identified in this study is shown. Restriction sites are abbreviated as follows: S, *Sma*I; X, *Xho*I; N, *Nco*I; E, *Eco*RI. Arrowheads represent transcription start sites and its directions of the IVa2 and MLP.

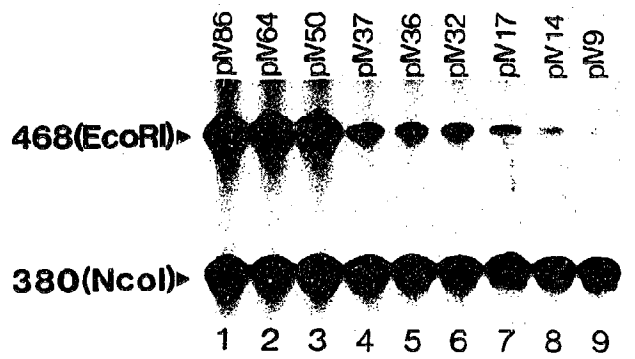

Fig. 2. 5′-Deletion analysis of the IVa2 promoter. Transcription activity of the pIV-series templates was analyzed in HeLa cell extracts as described in Materials and Methods. Run-off transcripts from *Eco*RI-cut experimental DNA (468 bases) and *Nco*I-cut reference pIV86 DNA (380 bases) are indicated by arrowheads.

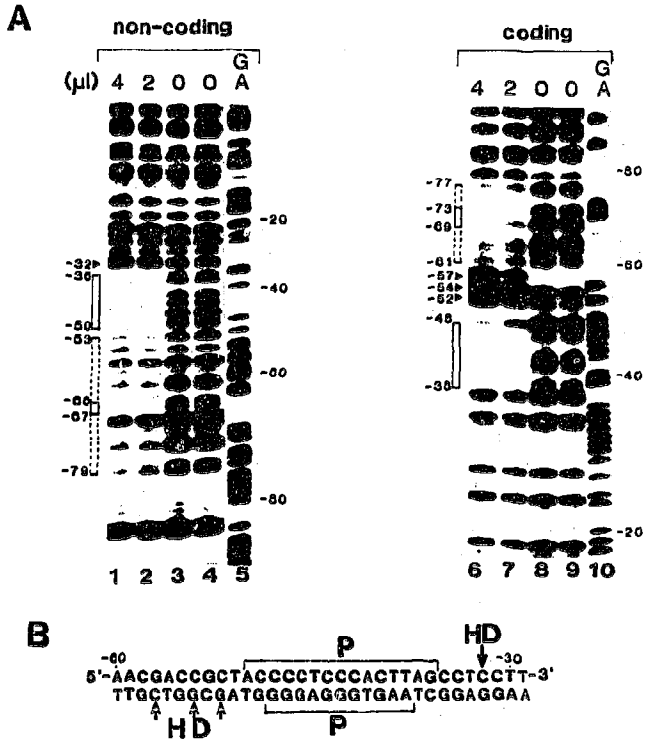

Fig. 3. DNase I footprint analysis of the IVa2 proximal promoter. (A) Footprint patterns around the proximal promoter region. The 5′-terminus at −159 and +51 was labeled with ^{32}P for the non-coding and coding strand probe, respectively. 0–4 μl of extract was used in each reaction. Strong (vertical boxes) and partial (broken vertical boxes) protections were shown beside the autoradiogram. Arrowheads = hyper-digested bands; GA = G+A ladders of a corresponding probe. (B) Schematic representation of footprint patterns around the strong protection. P = protection; HD = hyper-digested bands (arrows).

cient transcription from the IVa2 promoter is mediated by a cellular factor that interacts with sequences at least between −50 and −36. In this study, we designated this 15-mer sequences as a proximal promoter element (PPE) of the IVa2 promoter.

3.3. *The IVa2 proximal promoter element is equivalent to the GC-box*

We found that the PPE was enriched in C and G residues. To study binding specificity of PPE factor, we carried out competition footprint analysis (Fig. 4A). A competitor DNA fragment was mixed with the extract during the preincubation period, and then the coding strand probe was added. In our experimental conditions, pBR322 did not affect the PPE footprint. A *Xho*I-*Bam*HI fragment of pIV64 carrying the PPE competed out the binding, whereas a similar fragment of pIV37 did not affect the footprint (Fig. 4A, lanes 4 and 5). Neither DNA fragment carrying the upstream sequence of the MLP nor chicken conalbumin B promoter competed out the footprint (Fig. 4A, lanes 6 and 9). However, DNA fragment having SV40 21 bp-repeats: (GC-box) [12,13] and the herpes simplex virus thymidine kinase promoter (HSV-TK) [14,15] competed the footprint efficiently (Fig. 4A, lanes 7 and 8). These two heterologous DNA seemed to bind to a PPE factor more strongly than the homologous IVa2 sequence. These results suggest that there is a common sequence motif among PPE, SV40 GC-box and HSV-TK. Since the PPE is GC-rich (78%) and HSV-TK also contains Sp1-binding motifs [14,15], we regarded that the PPE is equivalent to the GC-box.

4. DISCUSSION

In this study, we demonstrated by in vitro analyses the presence of a *cis*-element at −50 required for efficient transcription from the IVa2 promoter and its cognate factor. Natarajan et al. [16] also investigated transcription element of the IVa2 proximal promoter in vivo, and demonstrated a *cis*-element at −49, CCCCTCCCACT. The PPE demonstrated in this study may be the same which Natarajan et al. reported. We suggested that the PPE can function in vivo.

We concluded that the PPE is equivalent to the GC-box binding factor for the following reasons. First, the factor bound to SV40 21-bp repeats without flanking sequences. Moreover, HSV-TK promoter also contains GC-boxes [14,15]. These two promoter sequences carrying 'standard' Sp1-sites competed PPE-binding factor stronger than the PPE itself. Second, the PPE is highly GC-rich, and a complementary inverted PPE sequence (CTAAGTGGGAGGGGT) showed a considerable homology with the consensus Sp1-binding motif ((T/G)GGGCGGPPY) [12,13] (Fig. 4B). Furthermore, promoters of HTLV-III (AIDS) virus [16] and the mouse dihydrofolate reductase (DHFR) genes [17] contain non-consensus Sp1-binding sites (Fig. 4B).

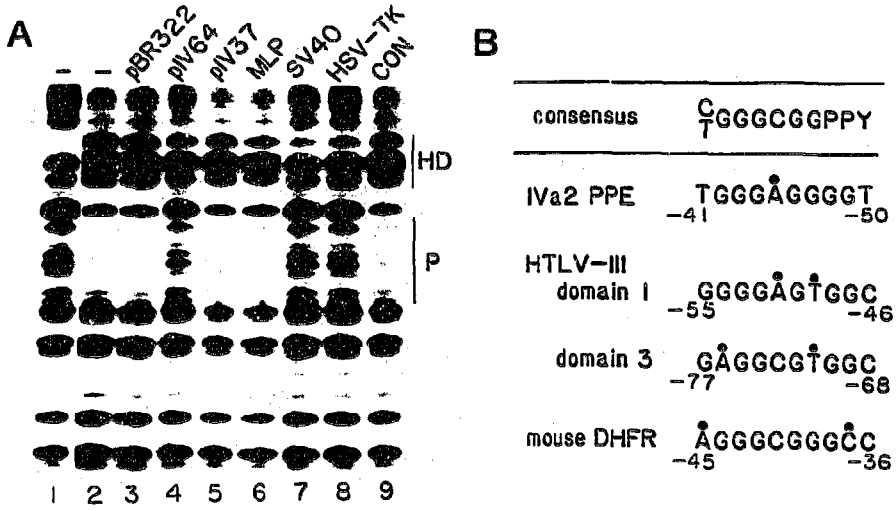

Fig.4. Binding specificity of the proximal promoter element-binding factor. (A) Competition footprint for PPE-binding factor was performed by using the coding strand probe. One picomole of DNA fragment carrying an upstream sequence of various promoters was used as a competitor. Used competitor DNAs are as follows: pBR322, pBR322 digested with *Bam*HI; pIV64, a *Bam*HI-*Xho*I fragment of pIV64; pIV37, a *Bam*HI-*Xho*I fragment of pIV37; MLP, MLP sequence between − 125 and + 33; SV40, SV40 sequence from 101 to 32 map position carrying the 21-bp repeats [22]; HSV-TK, herpes simplex virus thymidine kinase gene from −207 to −40 [22]; CON, the chicken conalbumin promoter from −120 to −44 [23]. − = no competitor. (Lane 1) Without extract; (lanes 2–9) with 1.5 μl of HeLa cell extract. Protected (P) and hyper-digested (HD) regions which are characteristically observed in the coding strand probe, are indicated. (B) Structural comparison of Sp1-binding sequences. Various Sp1-binding sites carrying an imperfect consensus sequence including the IVa2 PPE, human T-cell leukemia virus type III (AIDS virus) and the mouse dihydrofolate reductase genes were examined. Y and P mean pyrimidine and purine nucleotides, respectively. (•) bases which do not fit to the consensus.

Especially the GC-box in domain 3 of HTLV-III that has been demonstrated to function in the HTLV-III promoter showed fewer consensus points than the PPE. Though, at present time, we cannot conclude that PPE-binding factor is Sp1 itself, Sp1 may be the best candidate of a PPE-binding factor.

The IVa2 promoter does not have a typical TATA-box like cellular house-keeping genes, and accumulating results demonstrate that GC-rich sequence motifs often appear in their proximal promoters [18,19]. Little is known about mechanisms of transcription initiation of TATA-less promoters. In the TATA-carrying promoters, TFIID binds to the TATA-sequence and directs formation of preinitiation complex [3]. It is quite interesting that both TFIID [20] and Sp1 molecules [21] contain a long glutamine stretch. It may be possible that Sp1 can substitute for TFIID function to initiate transcription.

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