Cellular transcription factors that interact with p6 promoter elements of parvovirus B19

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All transcripts of the human parvovirus B19 identified so far are regulated by a single promoter at map unit 6 of the viral genome, the so-called p6 promoter. This promoter is active in a wide variety of different cells. In order to identify cellular transcription factors involved in regulating promoter activity, we performed gel-retardation and supershift assays using the parts of the p6 promoter sequence shown previously to be protected in footprint experiments. Thereby, binding was demonstrated of the Oct-1 protein to an octamer motif within the p6 promoter and of the transcription factor Sp1 to three GC boxes. A specific preferential interaction of the factor Sp3 with one of these boxes was observed, indicating that the ratio Sp1:Sp3 may be involved in the regulation of promoter activity. Consensus sites for the regulatory protein YY1 are located close to the GC boxes and the octamer motif, to which this factor binds efficiently.

Introduction

Parvovirus B19, the only member of the family Paroviridae known to be pathogenic in humans, is associated with a remarkable variety of diseases. It is, for instance, the causative agent of the common childhood disease erythema infectiosum (fifth disease) (Anderson et al., 1983) as well as acute and persistent arthritis. In addition, it is the cause of aplastic crisis in patients suffering from chronic haemolytic anaemia or hydrops foetalis after transplacental infection during pregnancy (Brown, 1984; Pattison et al., 1981; Reid et al., 1985).

At both ends of the 5’ to 6 kb single-stranded viral DNA genome, there are identical inverted terminal repeats of 383 nucleotides in length. The distal 365 nucleotides of these repeats are imperfect palindromes that form hairpin structures that are necessary to prime DNA replication. The only functionally active promoter within the viral genome, the p6 promoter, is located at the 5’ palindrome and regulates the synthesis of all nine viral transcripts (Blundell et al., 1987; Doerig et al., 1990). Seven of these are mRNAs and are used for synthesis of a multifunctional protein, the so-called non-structural protein 1 (NS1), two capsid proteins (VP1 and VP2) and several smaller polypeptides with no known function (Deiss et al., 1990; Luo & Astell, 1993; Ozawa et al., 1987).

Recently, the binding of Sp1 to a GC-box motif located downstream of an Ets-binding site (EBS) in combination with binding of human GA-binding proteins (hGABP) to EBS has been demonstrated (Vassias et al., 1998). The p6 promoter is highly active in different cell lines. Interaction of the virus NS1 protein with cellular transcription factors that have not been identified may increase promoter activity further (Gareus et al., 1998; Liu et al., 1991; Momoeda et al., 1994).

In this study, we characterized transcription factors from various cell lines and analysed their capacity to interact with the p6 promoter. Firstly, we tested the entire promoter region by electrophoretic mobility shift assays (EMSA) to get an overview of possible binding proteins. In a second approach, three promoter regions (region D, nt −219 to −187; region F, nt −133 to −92; region H, nt −76 to −35) that contain an increased number of potential binding sites for cellular
transcription factors and have been shown to be complexed with proteins (Liu et al., 1991) were selected for detailed study. Using these regions, we studied the interaction of DNA-binding proteins of three different nuclear extracts (from HeLa, K562 and BJAB cells) with the regulatory elements. Finally, these factors were characterized further by supershift assays with specific antibodies.

Methods

Oligonucleotides. Oligonucleotides containing the consensus binding sites for Sp1 and YY1 and respective unspecific oligonucleotides were obtained from Santa Cruz Biotechnology and were ready for use. Specific and mutated single-stranded oligonucleotides with consensus binding sites for hGAP, Oct-1 and oligonucleotides spanning regions D to H were obtained from Metabion and purified by HPLC. Annealing of the complementary oligonucleotides was performed by heating to 95 °C for 3 min followed by cooling to room temperature. The double-stranded oligonucleotides were radioactively labelled to a specific activity of ~100 c.p.m./μg by using T4 polynucleotide kinase and [γ-32P]ATP (5000 Ci/mmole; Amersham) and subsequently purified by chromatography (Micro Bio-Spin 6, Bio-Rad). Unlabelled double-stranded probes were used as specific and non-specific competitors in gel-retardation assays. The sequences of the eight oligonucleotides used in the band-shift assays were: region A, 5′-TAATCCGCGATCCGCGCCGGGCGGGACCAGTCTCGGCT; region B, 5′-TTCGGGTACAGATGCCGCCGGTCGCCGCCGGGTTAAGGGGACT; region C, 5′-TGCTGTAAGGCATTTCTGTGACCGGATGCACAGGAAATGACGTAATTGGTTTTG; and region H, 5′-TTCCTGCCCTTATGCAAATTGGCAGCCATTGCCGCCGGTCGCCGCCGGAGGTGGCGCTTACTATAATTTTATTGGTT-AGTTTTGTAACGGTCAAAATGGG.

Nuclear extracts. Preparation of nuclear extracts was performed at 4 °C; all buffers were placed on ice. Except for PBS, all buffers contained 0.5 mM dithiothreitol and 0.2 mM PMSF, added immediately before use. A modification of the method described by Dignam et al. (1983) for the preparation of nuclear extracts was used. HeLa or BJAB cells (1 × 10^9 cells/ml) were harvested (5 min, 750 g) and washed twice in PBS and once in hypotonic buffer (10 mM HEPES–NaOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂). The cells were pelleted (5 min, 750 g), resuspended in 1 vol. hypotonic buffer and incubated for 10 min on ice. The suspension was transferred to a Douche homogenizer and cells were lysed by a minimal number of strokes (20–30).

Nuclei were recovered by centrifugation (5 min, 750 g), suspended gently in 1 ml hypotonic buffer and pelleted (5 min, 750 g). The nuclei were suspended in 1 vol. (100–300 μl) high-salt extraction buffer (20 mM HEPES–NaOH, pH 7.9, 25% glycerol, 0.6 M KCl, 1.5 mM MgCl₂, 0.2 mM EDTA) and incubated for 30 min at 4 °C with gentle rocking. Following this incubation, the mixture was centrifuged (30 min, 100,000 g). The supernatant was dialysed at 4 °C for 3 h (10 mM HEPES–NaOH, pH 7.9, 10% glycerol, 100 mM NaCl, 1 mM EDTA). After dialysis, the extract was cleared by centrifugation (30 min, 100,000 g) to remove the precipitate. Aliquots of the supernatant were transferred quickly to liquid nitrogen and stored at −80 °C. The protein concentrations of the extracts were determined as 2 μg/μl by the Bradford method (Bio-Rad) with BSA as the standard.

K562 and BJAB cells (Fig. 1).

Electrophoretic mobility shift assays (EMSAs). Gel-retardation assays were performed with 2–5 μl nuclear extract of HeLa, K562 or BJAB cells. 1 μg poly(dI-dC) and 20 fmol labelled DNA in a final volume of 20 μl containing 8 μl binding buffer (100 mM Tris–HCl, pH 7.5, 500 mM NaCl, 5 mM dithiothreitol), 5 mM MgCl₂, 10% glycerol and 0.05% NP40. For controls, non-radio-labelled competitor oligonucleotides were added to the mixture prior to the addition of labelled DNA probes. Supershift assays were performed by adding specific antibodies to the reaction mixture. All binding reactions were performed for 20 min at room temperature. The samples were subjected to electrophoresis on native 5% polyacrylamide gels (37:5:1 acrylamide:bisacrylamide) containing 0.5% TBE. Gels were subsequently dried for autoradiography.

Antibodies. The polyclonal Oct-1-specific antiserum and monoclonal Sp1-, Sp3- and YY1-specific antibodies were obtained from Santa Cruz Biotechnology. Monoclonal antibodies directed to viral VP1 protein were used as a negative control (Gigler et al., 1999). In gel-mobility supershift experiments, 2 μl monoclonal antibody (2 μg/μl) or 10 μl Oct-1-specific antiserum (2 μg/μl) was added to the binding reaction after 20 min of incubation, which was then continued for additional 20 min.

Computer programs. Binding sites for transcription factors were analysed by using the program TFSEARCH 1.3 (http://pdp1.tcr.rwcp.or.jp/research/db/TFSEARCH.html) with the TRANSFAC matrix table 2.5 (Wingender et al., 2000).

Results

DNA-binding proteins of HeLa-cell extracts that interact with the p6 region

In order to characterize regulatory proteins that interact with the p6 promoter, we analysed the promoter sequence for the presence of DNA elements known to bind cellular transcription factors by using the TFSEARCH program. Within the promoter sequence, three regions were identified as containing elevated numbers of potential binding sites (region D, nt −219 to −187; region F, nt −133 to −92; region H, nt −76 to −35). Similar results were obtained in EMSAs with eight double-stranded oligonucleotides spanning the entire p6 promoter. Various cellular proteins could be identified that bind preferentially to these three promoter regions. These regions are furthermore highly conserved in various B19 isolates and have been shown for the most part to be protected by trans-acting proteins in footprint experiments (Liu et al., 1991). Based on these data, regions D, F and H were selected and used in detailed EMSA studies to test the binding of regulatory factors in nuclear extracts of HeLa, K562 and BJAB cells (Fig. 1).

With region D and nuclear extracts from HeLa cells, two major and two minor retarded bands were observed (Fig. 2A, lane 2). Region D showed two major retarded bands and one faint band (Fig. 3A, lane 2). Region H gave rise to four major and two minor bands (Fig. 4A, lane 2). Competition assays with unlabelled oligonucleotides demonstrated that all bands represented specifically binding proteins, since complex for-
Characterization of the B19 p6 promoter

Fig. 1. Schematic representation of the p6 promoter of parvovirus B19 with potential binding sites for cellular transcription factors. The position and sequence of the double-stranded oligonucleotides used in EMSAs (regions D, F and H) are given. Promoter regions that have been reported to be protected in footprint analysis (Liu et al., 1991) are marked by grey bars.

Fig. 2. Binding of cellular transcription factors to region D of the p6 promoter. EMSAs were performed with 32P-labelled oligonucleotides and 5 µg nuclear extracts prepared from HeLa cells (A) and from K562 and BJAB cells (B). After electrophoresis, the gels were exposed to X-ray film. The assignment of the complexes and the transcription factors that were identified as parts of the complexes are given. Lanes: 1, radiolabelled oligonucleotides; 2, radiolabelled oligonucleotides incubated with nuclear extracts (n.e.); 3, radiolabelled oligonucleotides incubated with nuclear extracts and specific (spec.) unlabelled oligonucleotides as competitors; 4, radiolabelled oligonucleotides incubated with nuclear extracts and unspecific (control) oligonucleotides; 5, 7 and 9 (competition shifts), radiolabelled oligonucleotides incubated with nuclear extracts from HeLa cells and specifically competing oligonucleotides (cons) containing the binding motifs for Sp1, EBS and YY1, respectively; 6, 8 and 10, radiolabelled oligonucleotides incubated with nuclear extracts from HeLa cells and unspecifically competing mutant oligonucleotides (mut) with substitutions in the binding sites for Sp1, EBS and YY1, respectively; 11 and 13 (immunoshift), radiolabelled oligonucleotides incubated with nuclear extracts from HeLa cells and control antibodies (α-C); 12 and 14, Radiolabelled oligonucleotides incubated with nuclear extracts from HeLa cells and antibodies specifically binding Sp1 (α-Sp1) and YY1 (α-YY1), respectively.
Fig. 3. Binding of cellular transcription factors to region F of the p6 promoter. EMSAs were performed as described in the legend to Fig. 2. Lanes: 1–4, as Fig. 2; 5 and 7 (competition shifts), radiolabelled oligonucleotides incubated with nuclear extracts from HeLa cells and specifically competing oligonucleotides containing the binding motifs for Oct-1 and YY1, respectively; 6 and 8, radiolabelled oligonucleotides incubated with nuclear extracts from HeLa cells and unspecifically competing mutant oligonucleotides with substitutions in the binding sites for Oct-1 and YY1, respectively; 9 and 11 (immunoshift), radiolabelled oligonucleotides incubated with nuclear extracts from HeLa cells and control antibody; 10 and 12, radiolabelled oligonucleotides incubated with nuclear extracts from HeLa cells and antibodies specifically binding Oct-1 and YY1 proteins, respectively.

In EMSA, both complexes II and III observed with region D were not formed in the presence of oligonucleotides corresponding to the EBS as competitors (Fig. 3A, lane 7). These data suggest that Ets-binding factors are parts of complexes II and III. We did not characterize these two complexes further because of the lack of appropriate antibodies. Recently, however, Vassias et al. (1998) identified GABPα and GABPβ, two members of the Ets family, as involved in the formation of these complexes. As the authors demonstrated, they correspond to heterodimers of GABPα/GABPβ1 and GABPα/GABPβ2, respectively.

The formation of complex I was blocked in the presence of unlabelled Sp1-consensus oligonucleotides (Fig. 2A, lane 5), indicating that Sp1 proteins are part of complex I. This was confirmed by immunoshift assays with Sp1-specific monoclonal antibodies (Fig. 2A, lane 12).

The analysis of region F showed that complexes VII and VIII were equivalent to complexes IV and V of region D. Competition analysis demonstrated that their formation was inhibited in the presence of unlabelled YY1-consensus oligo-
nucleotides (Fig. 3A, lane 7), whereby, in immunoshift assays with YY1-specific antibodies, the migration of complex VII in the gel system was reduced (Fig. 3A, lane 12). The formation of complex VI could be blocked by oligonucleotides containing the octamer binding motif (Fig. 3A, lane 5). Further analysis demonstrated that complex VI was supershifted after incubation with an Oct-1-specific antiserum (Fig. 3A, lane 10). In neither case did the use of unspecifically binding VP1-specific monoclonal antibodies show any effect on the intensity or mobility of the complexes.

Within region H, formation of complexes XIII and XIV was inhibited by YY1 consensus binding sequences (Fig. 4A, lane 7), whereas YY1-specific antibodies shifted only complex XIII (Fig. 4A, lane 15). This result is similar to that obtained from analysis of regions D and F. The intensities of the three complexes IX, X and XI were reduced by addition of unlabelled Sp1-consensus oligonucleotides, suggesting that Sp1 binds both of the GC boxes present in region H. Interestingly, the formation of complex XII was inhibited totally by these oligonucleotides (Fig. 4A, lane 5). Supershift assays with Sp1-specific monoclonal antibodies showed that Sp1 is involved in the formation of the upper three complexes (Fig. 4A, lane 9). Supershift experiments with Sp3-specific antibodies revealed that the transcription factor Sp3, which binds specifically to GC-box motifs with an affinity similar to that of Sp1 (Dennig et al., 1995; Hagen et al., 1994), is not only involved in the formation of complex XII but is also part of the upper three complexes, as indicated by the re-arrangement of these bands after addition of Sp3-specific antibodies (Fig. 4A, lane 11). The formation of all four complexes was inhibited by a combination of the two antibodies (Fig. 4A, lane 13).

In conclusion, we have shown that the transcription factor Sp1 interacts with three GC boxes located within regions D and H of the p6 promoter of parvovirus B19 and that the factor Sp3 binds preferentially to the GC box present in region H. Binding of Sp3 to the GC box in region D could not be demonstrated. It may be concluded that the ratio of Sp1 to Sp3 in the nucleus may regulate p6 promoter activity, as has been shown for several promoters controlling gene expression in various human cells (Hata et al., 1998; Nielsen et al., 1998). The

Fig. 4. Binding of cellular transcription factors to region H of the p6 promoter. EMSAs were performed as described in the legend to Fig. 2. Lanes: 1–4, as Fig. 2; 5 and 7 (competition shifts), radiolabelled oligonucleotides incubated with nuclear extracts from HeLa cells and specifically competing oligonucleotides containing the binding motifs for Sp1 and YY1, respectively; 6 and 8, radiolabelled oligonucleotides incubated with nuclear extracts from HeLa cells and unspecifically competing mutant oligonucleotides with substitutions in the binding sites for Sp1 and YY1, respectively; 9, 11, 13 and 15 (immunoshift), radiolabelled oligonucleotides incubated with nuclear extracts from HeLa cells and antibodies specifically binding Sp1, Sp3, both Sp1 and Sp3 (α-Sp1/Sp3; combination of two antibodies) and YY1, respectively; 10, 12 and 14, radiolabelled oligonucleotides incubated with nuclear extracts from HeLa cells and control antibodies (α-C).
Oct-1 protein forms a complex with the octamer motif of region F, the regulatory protein YY1 binds to the respective consensus sites located in all three promoter regions and an Ets-related transcription factor interacts with the EBS of region D.

**DNA-binding proteins in K562 and BJAB cells**

By analysis of the activity of the p6 promoter in different cell lines, enhanced promoter activity has been shown in the erythroid cell line K562 as well as in the B-cell line BJAB compared with epithelial HeLa cells (Gareus et al., 1998). For this reason, we were interested in studying nuclear extracts of K562 and BJAB cells by EMSA to detect additional transcription factors that regulate p6 promoter activity. In assays with region D, we obtained a pattern of bands similar to that of HeLa extracts (Fig. 2B). The main difference was that neither K562 nor BJAB nuclear extracts seemed to contain the degradation product YY1*. Besides the absence of YY1*, differences in complex formation were detected in region F with nuclear extracts of BJAB cells. In addition to the factors identified in nuclear extracts of HeLa cells, three further complexes could be identified (Fig. 3B, lane 2/BJAB). Complex c was characterized by immunoshift assay as the B cell-specific transcription factor Oct-2. Competition and immunoshift assays revealed that both complexes a and b consisted of two proteins (data not shown). Oct-1 and YY1 are involved in the formation of complex a, whereas complex b consists of Oct-2 and YY1. In assays containing extracts of K562 cells, an additional complex was formed with the oligonucleotide spanning region H (Fig. 4B, lane 2/K562).

**Discussion**

In order to define further the mechanisms involved in transcriptional regulation by the p6 promoter of parvovirus B19, we selected three adjoining regions within the promoter sequence to study the binding of cell-specific, trans-active nuclear proteins by EMSA. All the identified binding motifs within the p6 promoter are highly conserved in 19 isolates as analysed by DNA sequencing (data not shown). Since the isolates originated from patients with different parvovirus B19-correlated symptoms and were collected in different geographical regions, we speculate that these motifs are essential for promoter activity.

We could demonstrate binding of the transcription factor Sp1 to three GC boxes located in regions D and H as well as the binding of the Oct-1 protein, a lymphoid cell-specific factor, to an octamer motif within region F of the p6 promoter. Binding of Sp1 to the GC box adjacent to the octamer motif of region F could not be shown. This may be due to the fact that the dominant band of complex VI (Fig. 3A, lane 2), containing the Oct-1 protein, has the same mobility as the proposed complex with Sp1, and they would be superimposed. This hypothesis is, however, contradicted by the result of the supershift assay with Oct-1-specific antibodies, which did not reveal a co-migrating Sp1 complex. Also, incubation with unlabelled Sp1-consensus oligonucleotides or with Sp1-specific antibodies did not modify the migration of complex VI (data not shown). It may be concluded that the binding of Sp1 proteins is sterically inhibited by the factor Oct-1, which binds with high affinity to a binding site that partly overlaps the Sp1 motif. In addition to Sp1, both GC boxes located in region H interacted with the factor Sp3. The faster-migrating Sp3 complex XII (Fig. 4A, lane 2) could represent an amino-terminally truncated form of Sp3 containing a complete DNA-binding domain, as described previously (Hagen et al., 1992). Definite binding of Sp3 to the GC box of region D could not be demonstrated. These results indicate that different members of the Sp transcription factor family exhibit different modes of interaction with the GC boxes of the p6 promoter, suggesting additional influences of the adjoining nucleotide sequences. Alternatively, the ratio of Sp1 to Sp3, which has been shown to vary according to cell type and cell cycle phase, may affect the binding of the factors to the GC motifs (Kennett et al., 1997; Nielsen et al., 1998). In competition with Sp1, binding of Sp3 may repress or activate gene expression, as has been shown for several human genes (Hagen et al., 1994; Kwon et al., 1999; Qin et al., 1999) and virus genes (Tsai et al., 1999). Recently, Wildhage et al. (1999) have shown that three GC boxes located in the promoter controlling the expression of the human glucagon-like peptide-1 receptor do not bind Sp1 and Sp3 in equal amounts. Activator and/or repressor functions are thereby exerted that regulate the basal promoter activity of the receptor gene. Similar mechanisms may occur by interaction of factors Sp1 and Sp3 with the GC boxes of the p6 promoter.

YY1-binding motifs are located in all three promoter regions and YY1 proteins could be shown to form complexes with all of these sites. In region D, an EBS is located directly upstream of the GC box. Vassias et al. (1998) identified hGABP, an Ets-related transcription factor, as binding the CCGGAAGT motif formed by nucleotides −204 to −197, and suggested a synergistic effect on promoter activation of the formation of a complex between Sp1 and hGABP, an effect that was partially reversed by upstream binding of YY1.

It has been shown previously that the p6 promoter is about 25 times more active in the erythropoietin cell line K562 and six times more active in the B-cell line BJAB compared with its activity in HeLa cells. Cell-specific transcription factors may play an essential role in enhanced activation of the virus promoter. In BJAB cell extracts, we were able to identify the B cell-specific transcription factor Oct-2 as interacting with the octamer motif in region F. The additional complex that was observed in nuclear extracts of K562 cells, which are the most similar to the natural target cells of parvovirus B19 (Fig. 4B, lane 2), has yet not been characterized. This protein may be a cell-specific factor that contributes to the high activity of the p6 promoter in K562 cells. These factors must be identified...
before a final model for the regulation of parvovirus B19 gene expression can be proposed.

On the basis of the results presented here and the characterization of cis-active elements in the p6 promoter achieved by using reporter constructs (Gareus et al., 1998), we propose the following tentative model of activation by regulatory proteins. The binding of YY1 to at least two of the three binding sites facilitates the binding of further proteins to the DNA. This directs the binding of at least two Sp1/Sp3 molecules in the direct vicinity of the TATA box. Moreover, in the promoter region between nucleotides −125 and −195, an additional molecule of Sp1 and the Ets-related transcription factor hGABP may associate, whereas Oct-1 binds nucleotides −125 to −118. By bridging of the Sp1 molecules bound to regions D and H, a DNA loop may be formed, resulting in the initiation of transcription of the viral genes. As soon as the NS1 protein is synthesized, it may bind one of the molecules associated with the promoter, thereby mediating strong transcriptional activation and leading to significant enhancement of virus gene expression.

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