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YY1 is involved in the regulation of the bi-directional promoter of the Surf-1 and Surf-2 genes

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Abstract

The Surfeit locus is an unusual cluster of at least 6 housekeeping genes whose organisation is conserved between birds and mammals. We have previously shown that the divergently transcribed Surf-1 and Surf-2 genes are separated by a bi-directional, TATA-less promoter. In mouse, the Surf-1/Surf-2 promoter contains three important factor binding sites: Su1, Su2, and Su3. These sites are conserved between the mouse and human Surf-1/Surf-2 promoters, bind nuclear factors in vitro, and are required for accurate and efficient expression of Surf-1 and Surf-2 in vivo. Using gel retardation assays, methylation interference experiments, and specific antibodies we demonstrate that the Su1 binding factor is the initiator protein YY1. Over-expression of YY1 results in a major stimulation of transcription in the Surf-1 direction and a minor stimulation of transcription in the Surf-2 direction.

Key words: Bi-directional promoter; Housekeeping genes; Transcription initiation; YY1

1. Introduction

The initiation of transcription is a key control point in the regulation of gene expression. Initiation requires the formation of a complex protein–DNA assembly at the promoter ([1,2] and references therein). Promoters recognised by RNA polymerase II can be roughly divided into two classes: those with and those without a TATA box sequence [3]. To date, many of the well characterised promoters contain a TATA box. The TATA box is recognised by transcription factor TFIID and, for these promoters, the binding of TFIID appears to be the initial event in promoter recognition [2]. The second group of promoters (the TATA-less promoters) also require TFIID [4–6]; however, the details of transcription initiation at these promoters are less well understood. The initial event in the recognition of TATA-less promoters is thought to involve initiator proteins such as TF-I [7] and YY1 [8]. These proteins bind to DNA sequences (initiator elements) which generally overlap the transcription start point (reviewed in [9]). We are interested in the regulation of transcription initiation at the bi-directional, TATA-less promoter that lies between the Surf-1 and Surf-2 genes of the Surfeit locus [10,11].

The Surfeit locus is a tight cluster of at least six housekeeping genes (Surf-1 to Surf-6) unrelated by sequence homology [12,13]. Whilst most adjacent mammalian genes are separated by many kilobases, only a small number of base pairs separate any of the adjacent Surfeit genes. For example, in mouse the heterogeneous 5′ ends of the Surf-1 and Surf-2 genes are separated by a maximum of only 73 bp, the 3′ ends of the Surf-1 and Surf-3 genes are separated by only 70 bp, and the 3′ ends of the Surf-2 and Surf-4 genes overlap by 133 bp [12,10,14]. The Surfeit locus is also unusual in that the direction of transcription of each of the five characterised genes (Surf-1 to Surf-5) alternates with respect to that of its neighbour(s). In common with many other housekeeping genes, the 5′ end of each Surfeit gene is associated with a CpG-rich island [13]. The unusual organisation of this gene cluster is conserved between human, mouse, and birds and is, therefore, at least 300 million years old [15].

We have previously reported a detailed analysis of the DNA region in and around the transcription start sites of the divergent mouse Surf-1 and Surf-2 genes and have shown that it contains a bi-directional, TATA-less promoter [11]. Three important factor binding sites (Su1, Su2, and Su3) are present within this promoter region. These three factor binding sites are conserved between the mouse and human Surf-1/Surf-2 promoters, bind factors present in both mouse and human cell nuclear extracts, and are important for gene expression in vivo [11,16]. Mutation of the Su1 factor binding site results in decreased transcription in the Surf-1 direction but has little or no effect on transcription in the Surf-2 direction. Mutation of either the Su2 site or the Su3 site, on the other hand, inhibits transcription in both directions [11]. In order to understand the regulation of these genes, we are attempting to identify the factors which bind to these sites.

In this paper we show that the Su1 binding factor

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Abbreviations: bp, base pairs; PCR; polymerase chain reaction.

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corresponds to the initiator protein YY1. Over-expression of YY1 stimulates transcription in the direction of Surf-1 and, to a lesser extent, Surf-2.

2. Materials and methods

2.1. Proteins

Plasmid pHIS-YY1, which encodes human YY1 fused to the sequence MRGSHHHHHHGS at its N-terminal end, was kindly provided by T. Shenk [17]. Histidine tagged YY1 was purified from bacterial lysate by chromatography on a nickel (Ni^{2+}-NTA-agarose) column (Qiagen) essentially according to the method of Shi et al. [17]. Eluted YY1 was assayed for purity by SDS-PAGE followed by staining with Coomassie blue and quantitated using the Bio-Rad phosphoric acid protein assay (Bio-Rad). Polyclonal antibodies directed against YY1 were raised by immunising mice with the bacterially expressed protein. YY1-specific antibodies were affinity purified by chromatography on a YY1-Sepharose column produced using bacterially expressed YY1 conjugated to CNBr-activated Sepharose 4B (Pharmacia). HeLa cell nuclear extract was prepared as described by Wildeman et al. [18].

2.2. Gel retardation and methylation interference assays

Single-stranded oligonucleotides (100 ng) were 5'-end labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase. After annealing to the complementary oligonucleotide free label was removed using Sephadex G50 columns. Labelled oligonucleotides (10,000 cpm) were incubated with purified proteins (in the quantities indicated in the Figures) or 12 μg HeLa cell nuclear extract and 4 μg poly(dI-dC):poly(dC-dI) in binding buffer (12 mM HEPES pH 7.9, 5 mM $MgCl_2$, 60 mM KCl, 1 mM DTT, 50 μg/ml BSA, 0.5 mM EDTA, 0.05% NP40, and 10% glycerol). After 20 min at room temperature complexes were resolved on 5% non-denaturing polyacrylamide gels run in 1 × TBE. Methylation interference assays were performed using double-stranded, labelled oligonucleotides treated with dimethylsulphate (DMS) as described by Sambrook et al. [19]. The methylated probes (100,000 cpm) were incubated with 5 μg YY1 in the binding buffer described above. After 20 min at room temperature the complexes were resolved on non-denaturing gels and the bands excised and electroeluted. The DNA was then treated with piperidine as for Maxam and Gilbert sequencing and electrophoresed on 15% denaturing gels with Maxam and Gilbert size markers [19]. The sequences of the oligonucleotides used in this study have been reported previously [11,16].

2.3. Plasmids used in this study

The reporter plasmids used in this study are derivatives of pGL-basic (Promega). PCR was used to make a 207 bp *Hin*dIII-*Bgl*II DNA fragment carrying the human Surf-1/Surf-2 promoter. The template used for this reaction was cosmid C1, which has previously been shown to contain the human Surf-1/Surf-2 intergenic region [16,20]. The PCR primers used were:

5'-TGATCAAAGCTTCAGCCACCGCCGCCATCGCACC-3'

and

5'-TCTACGAGATCTCCGCAGAAACGCCCGCACGT-3'

These place a *Hin*dIII site 18 bp downstream of the major Surf-1 transcription start site and a *Bgl*II site 66 bp downstream of the major Surf-2 transcription start site. This fragment was cloned between the *Hin*dIII and *Bam*HI sites of pBlueScript KS$^-$ (Stratagene) creating plasmid pBS-H12 and its sequence verified using a Sequenase kit (USB) with alkali denatured, double-stranded preparations of plasmid DNA as template. To make a Surf-1-luciferase reporter fusion (pGL-HSurf-1) the Surf-1/Surf-2 region was excised from pBS-H12 using *Hin*dIII and *Kpn*I and cloned between the *Hin*dIII and *Kpn*I sites of pGL2-basic. To make a Surf-2-luciferase reporter fusion (pGL-HSurf-2) the Surf-1/Surf-2 region was excised from pBS-H12 using *Bst*YI and *Sac*I and cloned between the *Bgl*II and *Sac*I sites of pGL2-basic. Plasmid pGL-HSurf-1·Mut, which contains a mutated human Su1 (HSu1) factor binding site, was made using PCR-directed mutagenesis. PCR was carried out as described above except that the sequence of the *Hin*dIII oligonucleotide was:

5'-CAGCTAAGCTTCAGCCACCGCCGAAATCGCACC-3'

The underlined bases mis-match the template and produce the HSu1·M1 mutation [16]. The PCR product was cloned into pBlueScript KS$^-$ for sequencing then transferred to pGL2-basic on a *Hin*dIII-*Kpn*I fragment.

Human YY1 was over expressed in HeLa cells using the eukaryotic expression vector pMLVβplink [21]. PCR was used to obtain human YY1 on a *Bam*HI-*Cla*I fragment; the primers used were:

5'-AGCCCGAATTCATGGCCTCGGGCGACACCCTCTAC-3'

and

5'-TCTCTAAGCTTCACTGGTTGTTTTTGGCCTTAGCA-3',

with pHIS-YY1 as template. After cloning into pBlueScript the sequence of the PCR product was verified using a panel of specific sequencing primers. The sequenced clone was transferred on a *Bam*HI-*Cla*I fragment to pMLVβplink placing the expression of YY1 under the control of the human β-globin promoter activated by the MLV enhancer.

2.4. Transfection assays

HeLa cells were grown in 10% foetal calf serum (2×10^6 cells per 14 cm petri dish) before transfection with a total of 20 μg plasmid DNA by electroporation (270 V/960 μF). After 24 h, luciferase activity was determined using the Luciferase Assay System (Promega) according to the manufacturers instructions. The β-galactosidase expressing plasmid pRSV-βgal (5 μg) was included in each transfection as an internal control of transfection efficiency and the luciferase expressing plasmid pGL2-Control (Promega) was used as a positive control for the luciferase assay.

3. Results

3.1. YY1 is the Su1 binding factor

The Su1 factor binding site in the human and mouse Surf-1/Surf-2 bi-directional promoters has a similar sequence to the binding site of YY1, an initiator protein [22]. Furthermore, as expected for an initiator protein [9], the Su1 binding site overlaps the major Surf-1 transcription start point [11,16]. We reasoned that the Su1 binding factor could be YY1 or a related transcription factor. In order to determine whether YY1 was capable of binding to the Su1 site, histidine tagged YY1 protein was purified from bacteria containing plasmid pHIS-YY1 [17]. The HIS-YY1 fusion protein was purified by chromatography on a nickel column as described in section 2. Fig. 1A shows the results of a gel retardation assay in which recombinant YY1 protein was added to labelled DNA carrying the human Su1 (HSu1) factor binding site. After 20 min incubation at room temperature, protein–DNA complexes were separated from free DNA by electrophoresis on a 5% non-denaturing polyacrylamide gel and visualised by autoradiography. As can be seen from Fig. 1, a specific YY1-HSu1 complex (HSu1-YY1$_c$) is formed (marked by the arrow). At higher concentrations of YY1 protein a second retarded band of lower mobility is seen (marked by the arrowhead in Fig. 1A). This band may correspond to a YY1 dimer or other higher order complex. To investigate the specificity of the YY1–HSu1 complex we added competitor

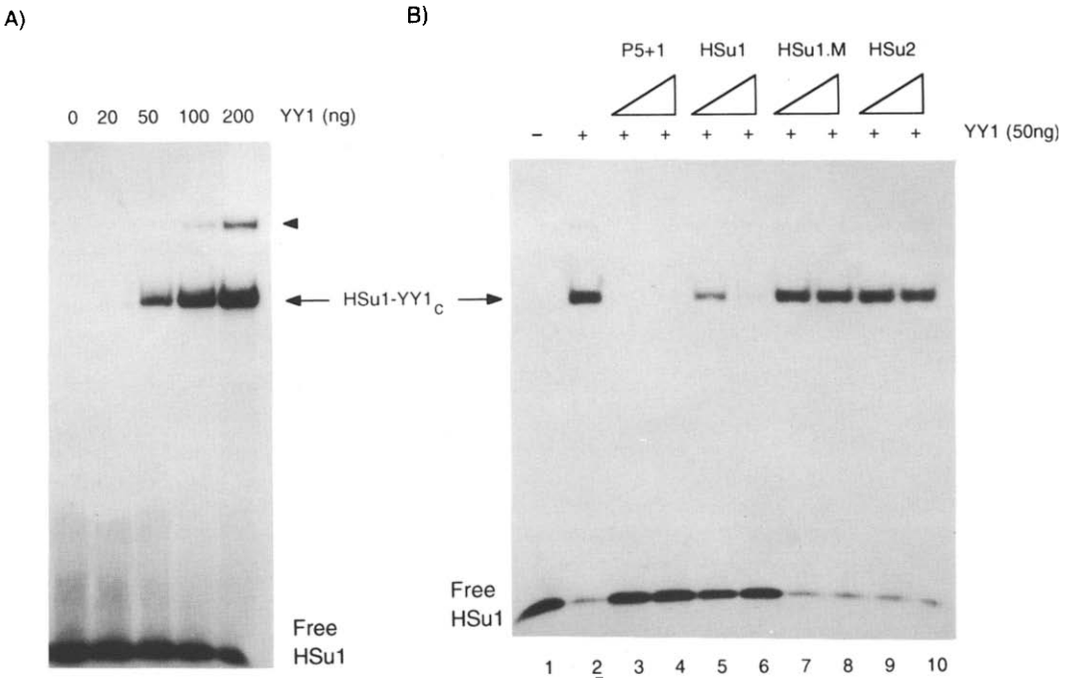

Fig. 1. YY1 binds to the human Su1 factor binding site. (A) Labelled oligonucleotides carrying the human Su1 (HSu1) factor binding site were incubated with the quantities of purified YY1 indicated, under the conditions described in section 2. The protein–DNA complexes were resolved on 5% polyacryamide gels and visualised by autoradiography. The primary HSu1–YY1 complex is indicated by the arrow, a lower mobility complex, which forms at higher YY1 concentrations, is indicated by the arrowhead. (B) The formation of the HSu1–YY1 complex was challenged by the addition of increasing amounts of competitor oligonucleotides (20 ng and 100 ng). The competitors indicated were added to labelled HSu1 prior to the addition of YY1.

oligonucleotides to the binding reaction. Addition of an oligonucleotide carrying the YY1 binding site from the adeno-associated virus (AAV) P5 promoter [17] abolishes the binding of YY1 to the HSu1 site (oligonucleotide P5 + 1; Fig. 1B, lanes 3 and 4). Similarly, the HSu1 factor binding site competes for the YY1–HSu1 complex (Fig. 1B, lanes 5 and 6). However, the HSu1 oligonucleotide is a less efficient competitor than the AAV P5 + 1 oligonucleotide, indicating that YY1 has a higher affinity for the AAV P5 + 1 sequence than for the HSu1 sequence. Competition with either a mutated HSu1 factor binding site (Fig. 1B, lanes 7 and 8) or the HSu2 factor binding site (Fig. 1B, lanes 9 and 10), has no effect on the YY1–HSu1 complex. These results are identical to those which we previously obtained with the Su1 binding factor present in mouse and human nuclear extract [11,16].

We have previously determined the position of G residues at which methylation by DMS prevents the binding of the Su1 binding factor present in nuclear extracts [11]. Using bacterially expressed YY1 we have now determined the G residues within the human Su1 site which are critical for YY1 binding (Fig. 2A). These results are summarised and compared to the results obtained using HeLa cell nuclear extract in Fig. 2B. As can be see from Fig. 2, YY1 and the Su1 binding factor present in HeLa cell nuclear extract have identical methylation interference patterns.

Further evidence to suggest that YY1 and the HSu1 binding factor are the same protein comes from studies using YY1-specific antibodies. Recombinant YY1 fusion protein was used to generate antisera in mice. Addition of YY1 antisera (or affinity purified YY1 antibodies) to reaction mixtures containing labelled HSu1 DNA and HeLa cell nuclear extract completely blocked the formation of the HSu1 complex (Fig. 3, lane 4). Pre-immune sera (data not shown) or antisera generated against an unrelated protein (SURF-5) had no effect on the formation of the HSu1 complex (Fig. 3, lane 5). Taken together, these results strongly suggest the Su1 binding factor present in mouse and HeLa cell nuclear extracts is YY1. Interestingly, the complex between bacterially expressed YY1 and HSu1 runs slightly slower on non-denaturing gels than the complex between YY1 from nuclear extract and HSu1 (compare lanes 2 and 3 in Fig. 3). This difference in mobility could be due to a post-translation modification of YY1 such as phosphoryation or to proteolytic degradation.

3.2. Over-expression of YY1 stimulates Surf-1 and Surf-2

We next determined the effect of YY1 over-expression on the human Surf-1/Surf-2 promoter in vivo in HeLa cells. The reporter plasmids used in these studies contain the human Surf-1/Surf-2 promoter directing the transcription of the luciferase gene and are shown diagrammatically in Fig. 4. Transcription in the Surf-1 direction

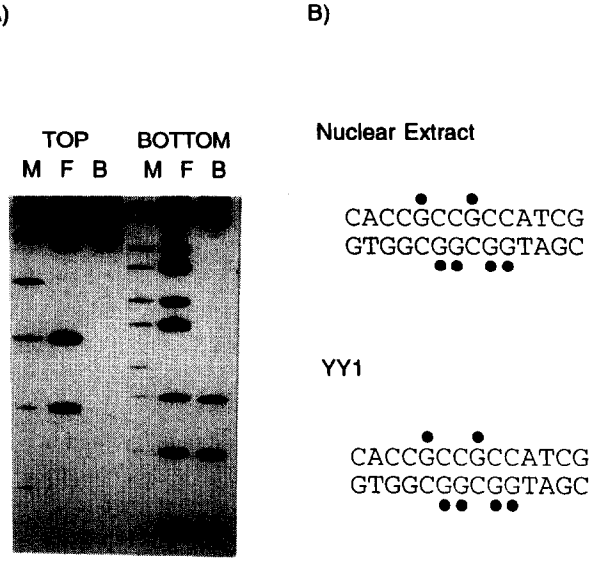

Fig. 2. YY1 and the Su1 binding factor have identical methylation interference patterns. (A) HSu1 oligonucleotides were labelled on either the top or bottom strands then methylated with DMS as described in section 2. After a 20-min incubation with recombinant YY1, protein–DNA complexes were resolved from free DNA by electrophoresis on a 5% non-denaturing gel. Free (lanes marked F) and bound (lanes marked B) DNA bands were excised from the gel and the DNA electro-eluted. After cleavage with piperidine, samples were run on a 15% sequence gel with Maxam and Gilbert sequence markers (lanes marked M). (B) The positions within HSu1 at which methylation by DMS prevents the formation of the HSu1–nuclear extract complex, and HSu1–YY1 complex, are indicated by dots.

was measured using plasmid pGL-HSurf-1 (Fig. 4B, line 1), whereas, transcription in the Surf-2 direction was measured using plasmid pGL-HSurf-2 (Fig. 4B, line 3). Human YY1 was cloned downstream of a constitutive promoter and co-transfected into HeLa cells with each reporter plasmid (see section 2 for details).

Transfection of the Surf-1-luciferase fusion into HeLa cells resulted in the production of significant luciferase activity (shown in Fig. 5, column 2). Presumably this activity is due to the presence of endogenous cellular factors which bring about transcription of Surf-1. Co-transfection of the Surf-1 reporter with the YY1 expressing plasmid resulted in an approximately 4-fold increase in luciferase activity (Fig. 5, column 3), indicating that YY1 can increase transcription in the Surf-1 direction. Transfection of the Surf-2-luciferase fusion into HeLa cells also resulted in the production of significant luciferase activity (Fig. 5, column 4). Again this activity is presumably due to the presence of endogenous transcription factors. Co-transfection of the Surf-2 reporter with the YY1 expressing plasmid resulted in a small increase (around 1.5-fold) in luciferase activity (Fig. 5, column 5). Our previous analysis of the mouse Surf-1/Surf-2 promoter showed that a mutation of the Su1 factor binding site reduced transcription in the Surf-1 direction by around 40% but did not measurably affect transcription in the Surf-2 direction [11]. Mutation of the human Su1 factor binding site, construct pGL-HSurf-1·Mut (shown in Fig. 4, line 2), also resulted in a minor but reproducible decrease in transcription in the Surf-1 direction (Fig. 5, column 6). Mutation of the human Su1 site prevented the activation of Surf-1 by co-transfected YY1 (Fig. 5, column 7).

4. Discussion

The Surf-1 and Surf-2 genes are divergently transcribed from a single, bi-directional promoter (shown diagrammatically in Fig. 4A). This head-to-head organisation has been observed for a number of other genes, some of which, for example the α1(IV) and α2(IV) collagen genes [23] and the avian GPAT and AIRC [24] genes, may be co-ordinately regulated. The Surf-1 and Surf-2 genes are transcribed in all tissues examined to date and therefore it is likely that they encode proteins with housekeeping functions. In common with the promoters of many housekeeping genes, the Surf-1/Surf-2 promoter is located within a CpG-rich island, has multiple transcription start sites (in both directions), and contains no sequences which resemble the consensus TATA box element [11]. As part of our study of the Surfeit locus, we

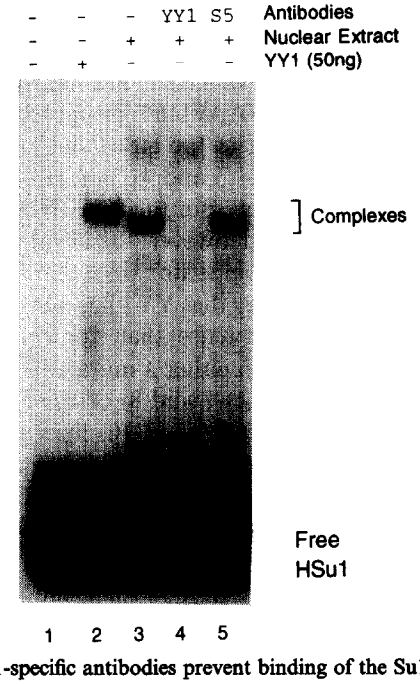

Fig. 3. YY1-specific antibodies prevent binding of the Su1 factor. Labelled HSu1 oligonucleotide was incubated with either recombinant YY1 (lane 2) or HeLa cell nuclear extract (lanes 3, 4 and 5). Protein–DNA complexes were resolved by electrophoresis on a 5% non-denaturing gel as described in Fig. 1. YY1 specific antibodies (added prior to the addition of nuclear extract) block formation of the nuclear extract factor–HSu1 complex (lane 4). Antibodies specific to an unrelated protein have no effect (lane 5). The YY1–HSu1 complex runs slightly slower than the nuclear extract factor–HSu1 complex (compare lanes 2 and 3).

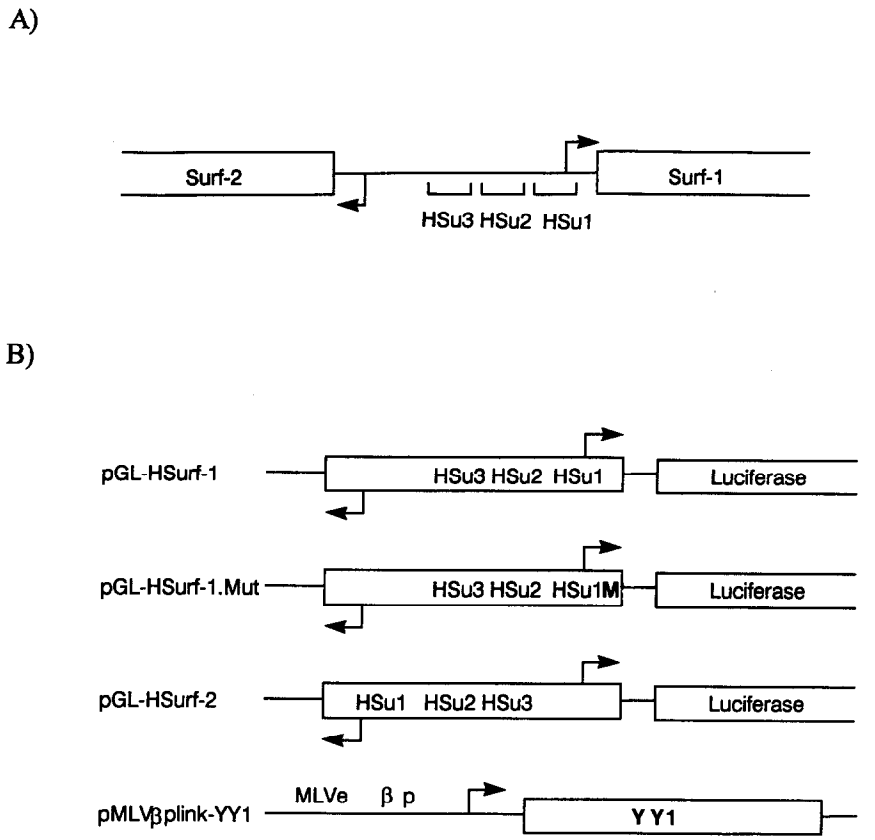

Fig. 4. DNAs used in this study. (A) The diagram shows the organisation of the human Surf-1/Surf-2 promoter region. The arrows represent the major transcription start points (for clarity the minor start sites are not shown). The arrangement of the HSu1, HSu2, and HSu3 binding sites is indicated. (B) The reporter plasmids used in this study are derivatives of pGL-Basic (Promega) and contain the Surf-1/Surf-2 bi-directional promoter (open box) cloned upstream of the luciferase gene. Transcription in the Surf-1 direction was measured using pGL-HSurf-1 (top line), whereas, transcription in the Surf-2 direction was measured using pGL-HSurf-2 (third line). The relative positions of the HSu1, HSu2, and HSu3 factor binding sites are indicated in each promoter. For clarity, only the major transcription start sites are shown. pGL-HSurf-1·Mut contains a mutation in the HSu1 factor binding site which prevents the binding of YY1 in vitro (see Fig. 1B). The construction of pMLVβplink-YY1 (bottom line) is described in the text. The human YY1 cDNA (open box) was cloned downstream of the human β-globin promoter activated by the MLV enhancer.

are particularly interested in two questions with regard to this promoter: first, in the absence of a TATA element, how is promoter recognition brought about, and second, is the transcription of the Surf-1 and Surf-2 genes co-ordinately regulated? As a first step towards solving these problems, we have attempted to identify the proteins which bind to, and regulate, this promoter.

The Su1 factor binding site present within the Surf-1/Surf-2 promoter overlaps the major transcription start point of the Surf-1 gene and has sequence similarity to the YY1 binding site. The transcription initiation factor YY1 has been implicated in the regulation of a wide variety of genes. In different promoter contexts YY1 appears to be capable of either positive or negative transcription regulation. For example, this protein has been shown to bind to a negative control element within the immunoglobulin k 3′ enhancer [25]. YY1 has also been shown to bind to a sequence present within the initial transcribed region of a number of ribosomal protein genes [22]. At least in the case of the rpL32 gene, which contains two YY1 (δ) factor binding sites, mutation of these sequences severely reduces promoter activity [26]. Finally, YY1 has been shown to bind to two sites within the adeno-associated virus P5 (AAV P5) promoter [17]. One of the YY1 binding sites within the AAV P5 promoter overlaps the transcription start point and mutation of this site severely reduces promoter activity [8].

Here we have shown that purified YY1 protein binds to the human Su1 factor binding site in vitro and produces the same methylation interference pattern as the Su1 binding factor present in nuclear extracts. We have also shown that YY1-specific antibodies block the binding of the Su1 factor present in nuclear extracts and that over-expression of YY1 stimulates transcription of Surf-1 in vivo. These data strongly suggest that YY1 is the Su1 binding factor and that YY1 acts positively, stimulating transcription of the Surf-1 gene. Surprisingly, the over-expression of YY1 also results in a modest stimulation of transcription in the Surf-2 direction. This implies that YY1 can function in a bi-directional manner, increasing

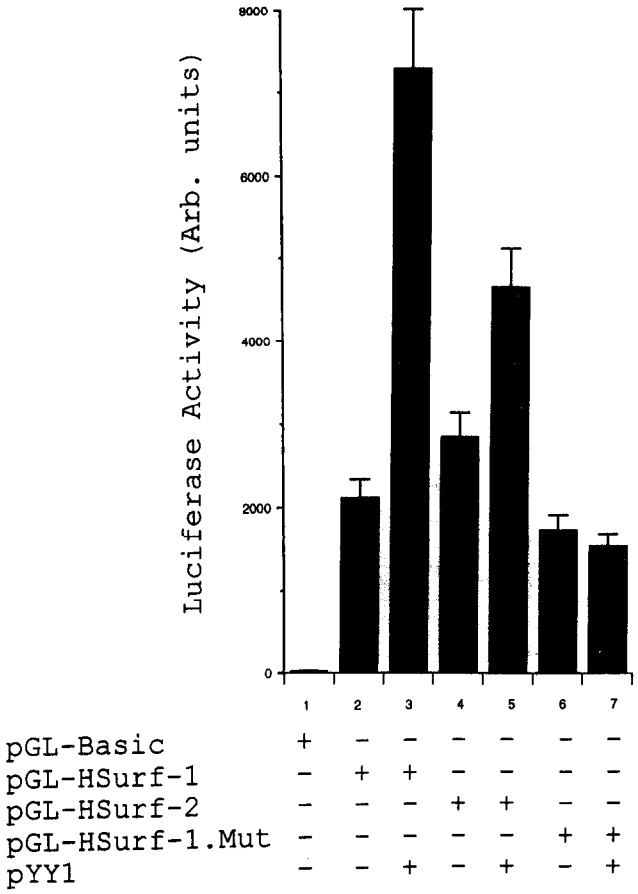

Fig. 5. YY1 can stimulate transcription of Surf-1 and Surf-2. The graph shows the levels of luciferase activity found in HeLa cell extracts 24 h after transfection with: (1) pGL-basic alone; (2) pGL-HSurf-1 alone; (3) pGL-HSurf-1 and pMLVβplink-YY1; (4) pGL-HSurf-2 alone; (5) pGL-HSurf-2 and pMLVβplink-YY1; (6) pGL-HSurf-1.Mut alone; and (7) pGL-HSurf-1·Mut and pMLVβplink-YY1. Enzyme activity has been normalised with respect to transfection efficiency using co-transfected pRSV-βgal. In each case values represent the average of five independent experiments.

transcription of both Surf-1 and Surf-2, and is evidence to suggest that transcription of Surf-1 and Surf-2 can be co-ordinately regulated.

We have previously shown that the Su1 binding factor is present in both HeLa cell and mouse L-cell nuclear extracts [11,16]. In keeping with this result, YY1 has been found in both human and mouse cells and is highly conserved [17,22,25]. Recent experiments by Shi and co-workers [27] and Shenk and co-workers [28] have shown that there is a physical interaction between YY1 and Sp1. In this context it is interesting to note that the human Surf-1/Surf-2 promoter contains a potential Sp1 binding site [16]. DNAse I footprinting has shown that the potential Sp1 binding site in the human Surf-1/Surf-2 promoter binds a factor(s) present in HeLa cell nuclear extract; however, this site is not conserved between the human and mouse promoters [16].

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