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Sp1 is essential for both enhancer-mediated and basal activation of the TATA-less human adenosine deaminase promoter

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ABSTRACT

Tissue-specific expression of the human adenosine deaminase (ADA) gene is mediated by transcriptional activation over a thousand-fold range. *Cis*-regulatory regions responsible for high and basal levels of activation include an enhancer and the proximal promoter region. While analyses of the T-cell specific enhancer have been carried out, detailed studies of the the promoter region or promoter–enhancer interactions have not. Examination of the promoter region by homology searches revealed six putative Sp1 binding sites. DNase I footprinting showed that Sp1 is able to bind these sites. Deletion analysis indicated that the proximal Sp1 site is required for activation of a reporter gene to detectable levels and that the more distal Sp1 sites further activate the level of expression. Inclusion of an ADA enhancer-containing fragment in these deletion constructions demonstrated that Sp1 sites are also essential for enhancer function. Apparently Sp1 controls not only low level expression but is also an integral part of the mechanism by which the enhancer achieves high level ADA expression. Mutagenesis of a potential TBP binding site at base pairs −21 to −26 decreased activity only two-fold indicating that it is not essential for transcriptional activation or enhancement.

INTRODUCTION

Adenosine deaminase (ADA) is an enzyme in the purine metabolic pathway which catalyzes the irreversible deamination of adenosine and deoxyadenosine to inosine and deoxyinsine respectively. It is expressed in all human tissues in a defined pattern over a thousand fold range (1–4). Among these tissues the highest levels are observed in thymus where it plays a critical role in T-cell survival by scavenging excess deoxyadenosine that is toxic to these cells. Its absence results in a form of severe combined immunodeficiency (SCID), a rare genetic disorder characterized by lack of T-cell function and impaired immune function (5,6). Unlike genes expressed in one tissue, or at one developmental time, which are regulated in an on–off fashion by binding of tissue or stage specific trans-acting factors, the ADA gene must accomplish differing levels of expression in multiple tissues as development and differentiation occur. Thus it provides an interesting model for a gene which is expressed in all cells, yet still can achieve activation to very high levels in some and expression at lower but highly modulated levels in others.

Regulation of ADA levels in human cells occurs mainly at the level of gene transcription (7). Transcriptional initiation presumably serves as the key point of regulation, although transcriptional arrest or pausing has been proposed as having a significant role in some cell types (8). The identification and characterization of the cis-regulatory elements and the trans-acting factors that interact to achieve appropriate spatial and temporal expression of the human ADA gene have been the recent focus of our research. Two functional DNA segments, a complex region from the first intron with T-cell specific enhancer activity and a proximal region upstream of exon one with promoter function, have been identified thus far. Characterization of the ~ 200 bp enhancer region revealed that a number of proteins functionally bind this segment (9). Analysis of these enhancer components is underway. The characterization of the human ADA promoter segment has been far less detailed. A 232 bp promoter segment from the 5′ end of the ADA gene has been shown to be effective in activating a chloramphenicol acetyl transferase (CAT) reporter gene to low basal levels in transient assay (4,10). High levels of CAT expression were obtained with this promoter in T-cells, in both transient assay and transgenic mice, in the presence of the ADA enhancer (4,9). However *in vivo*, in transgenic mice, the facilitator segments flanking either side of the enhancer are required for full, copy-number dependent, insertion-site independent expression of the transgene in thymus (9). The facilitators seem to assist in formation of the active chromatin region associated with the enhancer. A recent report indicates that the β-globin locus control region (LCR)/enhancer requires a functional promoter segment in transgenic constructions to consistently produce the chromatin activation associated with the LCR (11). Since the promoter of the human ADA gene has previously been only superficially

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characterized, we undertook a detailed delineation of its component parts. In this way we hoped to gain insight not only into basal promoter function, but also into what is required of the promoter elements for promoter/enhancer interactions and T-cell enhancer function.

Sequence analysis has previously identified six putative binding sites for the transcription factor Sp1 in the proximal region of the human ADA promoter (10,12). Similar Sp1 binding sites have been found in a wide variety of mammalian and viral promoters (13 and references therein). The functional nature of the putative Sp1 sites in the human ADA promoter has not been investigated. The examination of the human ADA promoter sequence also revealed a sequence similar to the consensus TATA binding protein (TBP) binding site that is situated in the proper location to bind that component of the basal transcriptional machinery (14). Our studies were designed to elucidate whether these elements or others were required for basal activation of the ADA promoter. Dissection of the promoter into its functional parts enabled us to determine which element(s) are required, which are supplementary, and which are unnecessary for basal activation. With this accomplished, we then investigated the relationship between other defined regulatory regions of the gene (e.g. the enhancer) and specific promoter elements which are required for enhanced activation of transcription. With this information, we can begin to understand the hierarchy of ADA regulatory components and start to design a basic model for the complex regulation of this gene.

MATERIALS AND METHODS

Preparation of ADACAT expression constructions

The previously described plasmid pADACAT 2.2 (4) was cut with NdeI, blunt-ended with Klenow and cut with BamHI to isolate the 3.8 kb fragment containing a 2.2 kb segment of the human ADA gene fused to the CAT coding sequence. This fragment was ligated into HindIII-cut, Klenow-blunted, BamHI-cut pUC18 plasmid DNA. The resulting plasmid was digested with HindIII and BssHII and the 4.6 kb fragment was blunt-ended with Klenow and recircularized to generate p18ADACAT. This plasmid contains an ADA promoter fragment from −2094 to + 97 cloned directly upstream of the CAT reporter gene. All other plasmids described are derived from it.

5′ truncations of the ADA promoter were generated by digesting p18ADACAT with BssHII and Bal 31 for various amounts of time. DNA was blunted with Klenow and dNTP's, HindIII-linkered and digested with BamHI and Hind III. The truncated 1.9 kb fragment was isolated and ligated to the 2.6 kb HindIII−BamHI fragment of p18ADACAT. The clones generated were sequenced to identify deletion break points. Selected clones were chosen such that putative promoter elements were partially or completely deleted.

Internal promoter deletions were generated in the following manner. A 4.58 kb ApaI fragment from p18ADACAT was recircularized to form Δ(71−34) with an internal deletion of Sp1 sites I, II and III. Larger internal deletions were generated from Δ(71−34) by digesting with ApaI and Bal31 for very limited lengths of time. DNA was blunt-ended with Klenow and digested with BamHI. The resulting fragments were both isolated and each was ligated to the corresponding ApaI (blunted)−BamHI fragment from Δ(71−34) such that unidirectional deletions from the ApaI site of Δ(71−34) were obtained in both directions. Δ(96−34) removes Sp1 sites IV and V, Δ(108−34) removes all of the remaining Sp1 sites, and Δ(71−10) removes the TBP binding site.

To create a specific mutation of the potential TBP site, a plasmid with an internal promoter deletion of 65 bp (Fig. 1, residues −74 to −10) was utilized. In this plasmid (not shown in Figure 5) a Not I site is serendipitously created at the deletion junction. The plasmid was digested with Not I and mung bean nuclease. A synthetic double-stranded oligonucleotide GGGCCCGGCCCGTCTGCAGGAGCGTGGCCGGCC was phosphorylated, ligated onto the blunt ends of the fragment described above and digested with PstI. A 4.6 kb fragment was isolated and self-ligated. Resulting plasmids were cloned and sequenced. A clone was isolated which had the oligonucleotide in the desired orientation. This plasmid was cut with ApaI and BssHII. The 4.5 kb fragment was isolated and ligated to a 172 bp ApaI-partial BssHII fragment from p18ADACAT to make p18ADACAT-T. This plasmid contains exactly the same sequence as p18ADACAT except that the TBP site TAAGAA has been replaced by a PstI site, CTGCAG, which has no homology to the TBP consensus binding sequence.

This original series of deletions was formed in a pUC18 plasmid backbone and gave questionable results upon transfection into cultured cells. To remedy this we chose to use the plasmid pBLCAT6 obtained from G.Schütz (15). Plasmids with the promoter truncations, deletions, or mutations described above were digested with either HindIII (5′ truncations) or BssHII (internal deletions and TBP mutation), blunt-ended with T4 polymerase and cut with NcoI. The 900−600 bp fragments of these digest were ligated to 3.7 kb XhoI (blunted)−NcoI fragment from pBLCAT6 recreating the series of deletions/mutations described above in the pBLCAT6 backbone. These clones were designated pADACAT211 and derivatives and are shown in Figs. 5 and 6.

An enhancer fragment was isolated from pSph2.3-, a pUC18 plasmid containing a 2.3 kb SphI fragment of the human ADA first intron enhancer (GenBank # 8327-10584) cloned into the SphI site of pUC18. This 2.3 kb fragment was removed by digestion with SalI and HindIII. Fragment ends were repaired with T4 polymerase and dNTP's, ClaI-linkered and subcloned into selected pADACAT211 promoter deletion/mutation plasmids at the ClaI site 3′ of the CAT reporter gene. This generated plasmids containing various promoter segments and the T-cell specific enhancer fragment downstream of CAT in its native orientaion (See Fig. 7). The enhancer fragment described was also cloned into pBLCAT6 to create a promotorless enhancer construction.

Plasmids were grown in DH5 α *E.coli* (Bethesda Research Laboratories) and purified by banding twice in CsCl gradients. All plasmids were sequenced by double-stranded dideoxy methods with Sequenase (U.S.Biochemical).

Transfection and CAT assays

A modified DEAE-dextran transfection protocol described previously (4) was used to introduce deleted plasmids into MOLT 4, an immature human T-cell line with high ADA levels (1650 nmoles/min/mg protein) or Raji, a human B-cell line with low ADA levels (14 nmoles/min/mg protein). Cell culture and CAT assays were done as previously described (4,9). All samples were transfected in duplicate. The resulting CAT activities for duplicate samples differed by less than 10%. The average value of the duplicates was used for the calculation of results. The absolute CAT activity for a particular construction, preparation of DNA,

or cell line transfected varied between experiments, probably as a result of transfection efficiency and cell state. However, the relative changes in CAT activity compared to controls were nearly identical.

Extract preparation

The plasmid, pSp1-516C, a generous gift from R.Tjian, was transformed into XL1-Blue *E.coli* (Stratagene) and bacterial extracts containing recombinant Sp1 were isolated as in Kadonaga (16). XL1-Blue extracts without the plasmid were made by the same method to serve as control. MOLT 4 nuclear extracts were prepared as described previously (9).

Electrophoretic mobility shift assay (EMSA)

A radiolabelled 128 bp Eco52 I–EcoRI fragment containing the human ADA promoter was prepared as for footprinting. 200 pg of this fragment was mixed with 3.6 μg of MOLT 4 nuclear extract in a volume of 25 μl at a final buffer concentration of 18% glycerol, 70 mM KCl, 33 mM Tris–HCl pH 7.9, 10.5 mM $MgCl_2$, 0.9 mM EDTA, 0.9 mM DTT, 0.5 mM $ZnCl_2$, 0.4 mM sodium metabisulfite, 80 μM PMSF, and 2 μg poly (dI-dC)-poly (dI-dC). 30 ng of a double-stranded Sp1 consensus oligonucleotide ATTCGATCGGGGCGGGGCGAGC or an Sp1 mutant oligonucleotide ATTCGATCGGTTCGGGGCGAGC (Santa Cruz Biotechnology) were added to the EMSA reaction with the labelled probe as competitors. For antibody supershift analysis, 3.6 μg of MOLT 4 extract was incubated overnight at 4°C with 5 μg of rabbit polyclonal IgG to either Ets-1/Ets-2 or Sp1 (Santa Cruz Biotechnology). Extract–antibody mixtures were then used for EMSA. Complexes were separated by electrophoresis through 3.8% non-denaturing polyacrylamide gels at 4°C, 20 mA in 25 mM Tris base, 190 mM glycine, 1 mM EDTA.

DNase I footprinting

Fragments used for footprinting were isolated from pADA-CAT211. Either a 233 bp Eco52 I–BamHI fragment or a 128 bp Eco52 I–EcoR1 fragment was used to generate the upper strand footprint. The lower strand was footprinted with a 270 bp BamHI–BsaI fragment. Klenow, [α-^{32}P] dGTP (3000Ci/mmole; NEN) and dNTP's were used to fill in the Eco52 I and BamHI site respectively after digestion of pADACAT211 with these enzymes. Plasmids were then cut with the second enzyme and the fragment of interest was isolated from low melt agarose by melting, phenol extraction, and precipitation. DNase I footprinting was performed as described previously (9) using 0–20 μg of crude bactrial extract containing rSp1 or 0–18 μg of MOLT 4 crude nuclear extract.

RESULTS

Analysis of ADA promoter

A 232 bp segment from the 5′ end of the human ADA gene has been shown previously to be effective at activating a CAT reporter gene in transient assay in a number of different cell types (4,10). In transgenic mouse systems this promoter segment is also capable of driving ubiquitous tissue expression of the CAT reporter, as well as high level thymic CAT expression (9). This pattern of transgene expression was only observed from constructions that also contain a fragment encompassing the ADA first intron enhancer region. A segment of this promoter is shown in Fig. 1. Like many genes with GC rich promoters, the ADA gene has multiple transcriptional start sites. One major and several minor start sites have been identified in both mouse (17–19) and human (10) by several different techniques. Sequence analysis of this DNA fragment from the human gene revealed 6 potential Sp 1 binding sites (10,12). These sites, labelled I–VI, are shown in Fig. 1. Also shown is the location of a potential TBP binding site, TAAGAA, and the major transcriptional start site.

An homology comparison between the murine and human ADA promoter regions has been published previously (20). Limited regions with significant homology were identified, mostly due to the GC rich nature of both promoters. Unlike the proximal promoter of many other genes, this area does not seem to be highly conserved between the species. The functional elements appear similar but not identical. The murine gene has five non-overlapping Sp1 sites whose sequence and position relative to the major transcriptional start site differ significantly from the six overlapping Sp1 binding sites in the human promoter. The murine gene also has a recognizeable TATA motif, TAAAAAA, which has been shown to bind to the TFIID fraction (21). The human gene sequence at that location, TTAAGAA, is more divergent from the canonical TBP binding sequence (22). It has been questioned whether or not the TAAGAA sequence might function in TBP binding (12). In fact, the human adenosine deaminase gene is often cited as containing a TATA-less promoter (10,13,23,24).

Electrophoretic mobility shift assay (EMSA)

The ability of a small 128 bp fragment of the human ADA promoter to bind protein(s) in MOLT 4 nuclear extracts was assessed by EMSA and is shown in Figure 2. Formation of the shifted complexes designated by small arrowheads (Fig. 2, Lane 2) was inhibited with an excess of an unlabelled oligonucleotide containing an Sp1 consensus binding site (Fig. 2, Lane 5) but not a similar amount of an oligonucleotide containing a mutation in the Sp1 site (Fig. 2, Lane 6). Addition of a polyclonal antibody against Sp1 resulted in a reduction of the intensity of the of the Sp1 specific bands and the concomitant appearance of a supershifted band (shown by the large arrowhead in Fig. 2, Lane 3) indicating the presence of Sp1 in these complexes. The addition

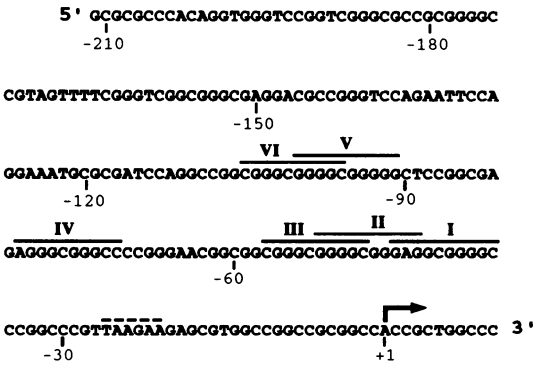

Figure 1. Human ADA promoter sequence. The sequence proximal to the ADA major transcription start site (+1), indicated with the bent arrow, is shown from −211 to +11. This corresponds to the segment from 3725 to 3946 in the GenBank numbering for the human ADA gene. This promoter lacks a canonical CCAT box sequence. The sequence from −21 to −26, indicated with a broken line, has low homology to the TATA binding protein (TBP) consensus binding site and is in the proper location to bind TBP. Six consensus matches for the trans-acting factor Sp1 are shown beneath the solid lines and are labelled I–VI.

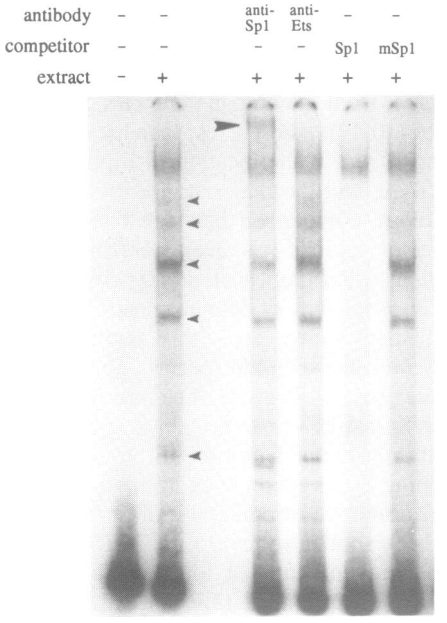

Figure 2. Electrophoretic mobility shift assay (EMSA) of the human ADA promoter with MOLT 4 extract. Labelled probe was made as described. 200 pg was incubated alone (Lane 1) or with 3.6 μg of MOLT 4 nuclear extract (Lane 2). Binding competition was performed with an 800-fold molar excess of an oligonucleotide containing one consensus Sp1 binding site (Lane 6) or an identical amount of an oligonucleotide containing a mutated Sp1 site (Lane 7). 3.6 μg of MOLT 4 extract was also incubated overnight at 4°C with either 5 μg of polyclonal rabbit anti-Ets-1/Ets-2 IgG (Lane 4) or 5 μg of polyclonal rabbit anti-Sp1 IgG (Lane 3). Labelled probe was then added and allowed to incubate at 20°C. Complexes were separated by electrophoresis through non-denaturing polyacrylamide gel at 4°C. Small arrowheads denote shifted complexes which can be competed with an Sp1 oligonucleotide but not the mutant. Antibody supershifted complex is indicated with the large arrowhead.

of a polyclonal antibody to the Ets family of transcription factors resulted in no bands with reduced intensity or supershifted bands (Fig. 2, Lane 4).

Footprinting analysis

The sequence and position of the consensus Sp1 binding sites within the human ADA promoter are shown in Fig 1. All sites possess the GGGCGG core binding sequence characteristic of GC boxes with the exception of site I which has the sequence AGGCGG. Homology to the Sp1 consensus binding site, [(G/T)(G/A)GGCG(G/T)(G/A)(G/A)(C/T)] (25), varies from 80−100%. Elements with sequence identical to sites I and V in the ADA gene promoter have been shown to bind Sp1 in the context of other promoters where they were found (23,26,27). The ability of the remaining sites to bind Sp1 was unknown. In order to assess this ability and to determine relative affinities of these sites for Sp1, DNase I footprinting was performed. A recombinant Sp1-*lac* Z fusion protein from pSp1-516C (16) containing the Sp1 DNA binding and activation domains was produced in bacterial cultures and extracts were used to footprint radiolabelled ADA promoter fragments containing all six putative Sp1 binding sites. All potential Sp1 sites are protected from DNase I digestion on both strands by pSp1-516C extract (Fig. 3). XL1-Blue bacterial extract without Sp1 did not footprint this region (data not shown).

The footprinted region produced with MOLT 4 nuclear extract (shown in Fig. 4) varies from that generated with bacterial extract

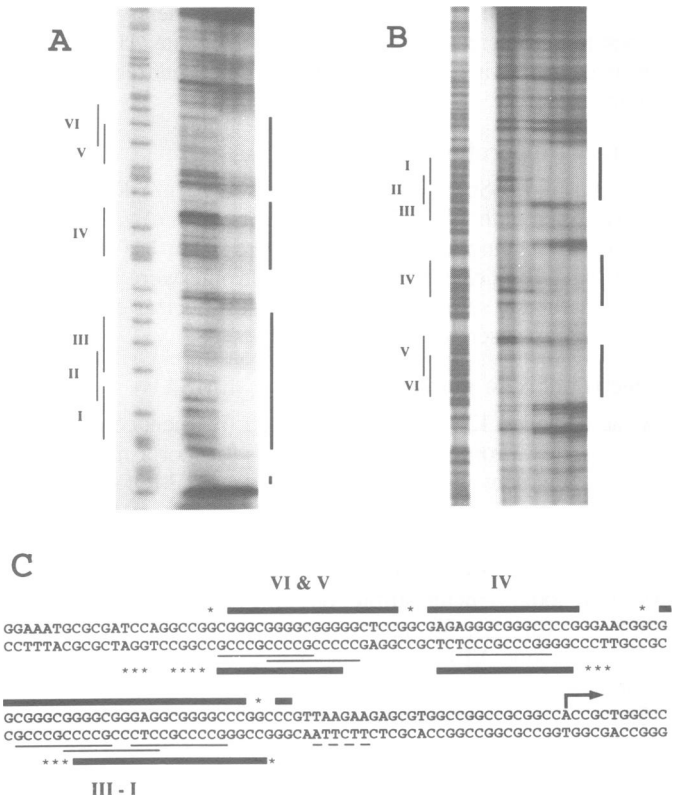

Figure 3. DNase I footprinting of the human ADA promoter with recombinant Sp1 (rSp1). Fragments for footprinting were generated from pADACAT211 and labelled as described in the methods. Bacterial extracts containing rSp1 were incubated with end-labelled fragment and limited DNase I digestion was performed. The resulting fragments were separated on 6% sequencing gels. Locations of the Sp1 sites I−VI are marked with thin solid lines. Protected regions are indicated with heavy lines. **(A)** A 233 bp Eco52I−BamHI fragment was used for generating the coding strand footprint. Lane 1 is a Maxam−Gilbert C+T sequencing ladder of the fragment. Lane 2 contains no bacterial extract and lanes 3−5 contain 5μg, 10μg, and 15μg, respectively, of bacterial extract containing rSp1. **(B)** A 270 bp BamHI−BsaI fragment was used to footprint the non-coding strand. Lane 1 contains the C+T ladder for the fragment and lanes 2−6 contained 0μg, 5μg, 10μg, 15μg, and 20μg of bacterial extract. **(C)** Protected areas and consensus Sp1 sites are indicated relative to the promoter sequence. Increased sensitivity of a site to DNase I digestion is marked with an asterisk *.

containing recombinant Sp1. At intermediate levels of extract, the entire proximal promoter region from site IV to beyond the transcription start site becomes DNase I resistant (Fig. 4, Lane 7). This may be due to additional proteins that are able to interact either with this region of the promoter or with the bound Sp1 proteins. The most notable difference with the crude nuclear extract is the absence of a protected region over sites V and VI even at the highest protein levels examined. This may be relevant to the results of the deletion analysis described below.

Promoter deletion analysis

In order to assess the functionality of these Sp1 sites in a transient assay system, the plasmid pADACAT211 was made, along with various 5′ truncations and internal deletions of the promoter segment affecting the Sp1 sites and the potential TBP site (Fig. 5). These plasmids were tested for their ability to activate the CAT reporter gene after DEAE−dextran transfection into the human lymphoid cell lines MOLT 4 and Raji. These cell lines have both been shown to efficiently utilize the intact human ADA

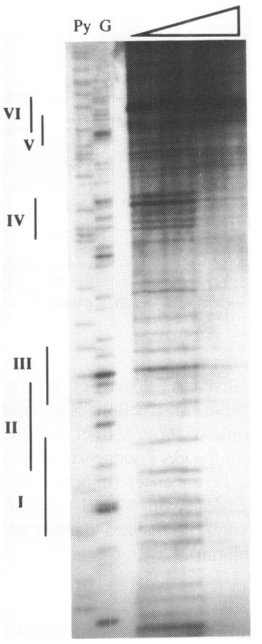

Figure 4. DNase I footprinting of the ADA promoter with MOLT 4 extracts. Footprinting was performed as described for Fig. 3 using MOLT 4 nuclear extracts. Sp1 sites are indicated as in Fig. 3. Maxam–Gilbert C+T and G sequencing ladders are shown in Lanes 1 and 2. Lanes 3–9 contain 0 μg, 0.9 μg, 1.8 μg, 4.5 μg, 9 μg, 12.6 μg, and 18 μg respectively.

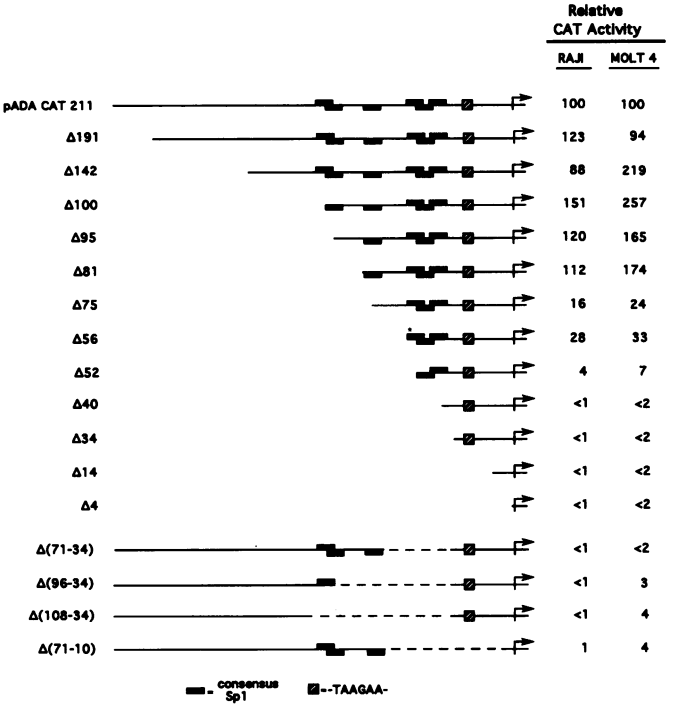

Figure 5. CAT activity of transfected promoter deletions. The structure of the proximal promoter region of Bal 31-deleted constructions from the pADACAT211 series are depicted graphically in Fig. 5. The constructions with 5′-truncations of the promoter are designated with a Δn where 'n' is the length of the remaining promoter segment. The constructions with internal deletions are designated by indicating the negative numbers corresponding to the residues immediately adjacent to the deleted segment. Sp1 sites are indicated by filled boxes, the TBP binding site by a hashed box, the major transcription start site with the bent arrow, and internal deletions with a broken line. Sp1 sites shown represent those indicated as sites I–VI (right to left) in the text. These constructions were transfected into MOLT 4 or Raji cells. Cell extracts were prepared and assayed for CAT activity. The activities were normalized to that obtained for the parental construction, pADACAT211 (at the top of Fig. 5), which was set at 100. In clone Δ56 the first C of Sp1 site III was deleted and the juxtaposed G (*) results in an improved consensus in the Sp1 site of GGGGCGGGGC.

promoter in this transfection-transient assay system (4). Cell extracts were prepared 48 hours after transfection and were assayed for CAT activity. The original series of deletions was formed in a pUC18 plasmid backbone and gave anomalous results upon transfection into cultured cells. Removal of promoter elements was not sufficient to destroy CAT expression. This seemed to be due to cryptic transcriptional starts within the plasmid which severely interfered with interpretation of results. Previously in our lab, similar results (unpublished data J.C.States, D.A.Wiginton, and J.J.Hutton) had been seen using the pSVO-CAT vector backbone (28). To remedy this we chose to use the plasmid pBLCAT6 (15), in which the plasmid sequences responsible for cryptic starts have been deleted and replaced with two polyadenylation signals upstream of the 5′ multiple cloning site. This efffectively prevents upstream starts, as evidenced by the fact that the promoterless parent vector had no activity in any cell lines tested. DNA segments from the parental plasmid and all subsequent deletions were sublcloned into pBLCAT6 as described in the methods to generate pADACAT211 and the pADACAT deletions shown in Figs. 5 and 6.

Relative CAT activities obtained after transfection of these plasmids are also shown in Figs. 5 and 6. Values for CAT have been normalized to the parental construction (top in each figure). The deletion of sites V and VI (Fig. 5, Δ100 and Δ95) has little effect on resulting CAT activity. This correlates well with the absence of a DNase I protection footprint over these sites using MOLT 4 extracts and suggests that these sites may not bind Sp1 *in vivo* even though they have the capacity to do so. Deletion of site IV (Fig. 5, Δ75) reduces activity 6–7 fold in both MOLT 4 and Raji. Deletion of site III (Fig. 5, Δ52) reduces activity another 3–4 fold. Converting site III into a better consensus Sp1 sequence, GGGGCGGGGC (Fig. 5, Δ56) by replacing the first residue (C to G) increases activity slightly relative to Δ75. Sites I and II were not separated by any of the deletion clones we obtained, but their simultaneous deletion (Fig. 5, Δ40 and Δ34) results in loss of detectable CAT activity in either cell line. These results would indicate that sites I and/or II alone are capable of low level basal activation of the promoter, and addition of sites III and IV further activates transcription to its maximal unenhanced level. The addition of sites V and VI do not appreciably increase activation above this level in our assay system.

Interpretation of the results of the internal deletions is less straightforward than the truncations. Each of these constructions is inherently different from the parental construction in the placement of the remaining Sp1 elements relative to the transcriptional start site. Internal deletions Δ(71–34) and Δ(96–34) maintain the spacing between the transcriptional start site and the most proximal remaining Sp1 site at a distance near that found in the endogenous ADA gene (–36 bp), although the sequence for the Sp1 site found in this position varies. An internal deletion removing sites I–III [Fig. 5, Δ(71–34)] placed site IV at –35 bp, a distance almost identical to the location originally

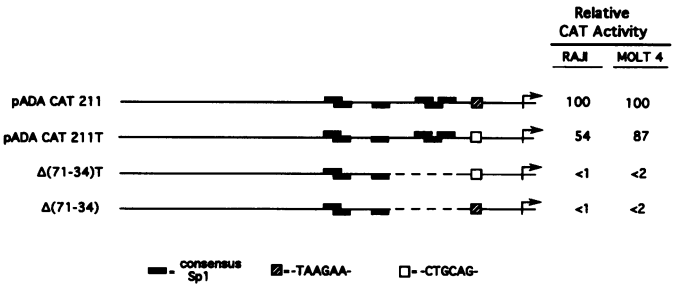

Figure 6. CAT activity of transfected TBP mutants. Devices used to depict promoter elements are as in Fig. 5, with the addition of an open box representing the mutant sequence, CTGCAG, whose presence is designated by the addition of T to the name of the construction. This has been substituted in some clones for the native sequence TAAGAA, depicted with the hashed box. Resulting CAT activity is shown normalized to pADACAT211 which was assigned a relative value of 100.

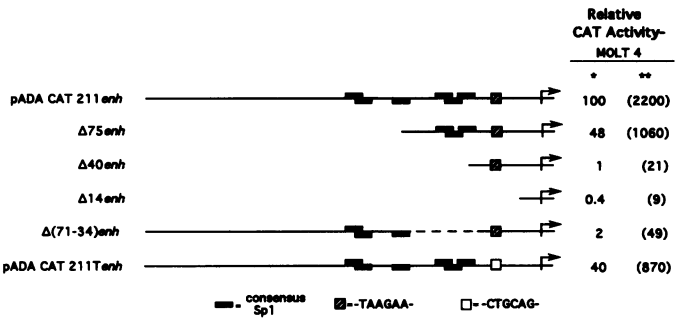

Figure 7. Structure and relative CAT activity of promoter–enhancer constructions. The promoter region for these constructions is as shown above. These promoter regions are identical to some of those shown in Figs. 5 and 6. These plasmids also contain, downstream of the CAT sequence, a 2.3 kb ADA intronic fragment that contains a T-cell specific enhancer (indicated by the addition of enh to the names). Transfections were done with MOLT 4 cells only and the CAT activities are reported two different ways: normalized to either pADACAT211enh (*column) or pADACAT211 (** column).

occupied by site I. This construction was unable to activate transcription to a detectable level in either cell line even though it has three Sp1 sites (the same number as Δ75), the closest of which is the same relative distance from the transcriptional start site as sites I/II in the parental construction. This implies that these Sp1 sites, IV–VI, are functionally different in their ability to substitute for the more proximal sites, I–III, and activate transcription. A larger internal deletion positioning site VI alone [Fig. 5, Δ(96–34)] at −35 bp results in very low level activation at best. Deletion of the putative TBP binding site [Fig. 5, Δ(71–110)] moves sites IV–VI to −11 bp, −31 bp, and −36 bp respectively. This construction also produces very low CAT activity. The overall results also demonstrate the absence of other more distal sequences which are necessary or sufficient for activating transcription.

Mutation of the potential TBP site

The sequence TAAGAA is found in the ADA promoter at −21 to −26 bp relative to the major transcription start site at the proper location to function in the binding of TBP. A similar sequence, TAAAAAA, is present in the murine ADA gene at −21 to −27 bp and has been reported to be essential for promoter function (21). In order to determine the necessity of a similar sequence at that site for activation of the human ADA gene, it was mutated by clonal manipulation from TAAGAA into a PstI site, CTGCAG [Fig 6, pADACAT211T and Δ(71–34)T] and tested in transient assay by transfection into MOLT 4 and Raji cells. This mutation alone did not obliterate activity, although a small decrease in activation of 50% or less was observed in both Raji and MOLT 4. While modification of this sequence has a discernible affect, this specific site is not essential for promoter function.

Promoter requirements for enhancer function in MOLT 4

The ADA T-cell enhancer was included in selected plasmids (shown in Fig. 7) downstream of the CAT coding sequence in its natural orientation relative to the promoter. The enhancer has been shown previously to be utilized very efficiently in MOLT 4 but not Raji (4). Therefore, promoter-enhancer constructions were transfected only into MOLT 4 cells to examine which promoter elements, if any, are required for enhancer driven activation. The plasmid pBLCAT6enh, which contains the enhancer but lacks the ADA promoter, showed no detectable activity (data not shown). Inclusion of the ADA transcription start (Fig. 7, Δ14enh) gave activity at very low but detectable levels. Addition of the 'TBP' site (Fig. 7, Δ40enh) increased activity an additional two-fold. The level of activation seen in the presence of the enhancer and Sp1 sites reflects a further 50–100 fold increase in CAT activity over Δ40enh (Fig. 7, pADACAT211enh and Δ75enh). All the constructions containing the enhancer show a 20 to 50 fold increase above the equivalent plasmids lacking the enhancer. Results of this experiment show that the enhancer is capable of recognizing and activating the truncated promoter (Fig. 7, Δ40enh and Δ14enh) to low levels even in the absence of Sp1 sites. However, higher levels of activation require the presence of both the enhancer and the Sp1 binding sites.

The TBP mutant pADACAT211Tenh shows a 60% decrease in activity from pADACAT211enh. This is very similar to the results seen in the absence of the enhancer, demonstrating that neither the Sp1 nor the enhancer activated transcription has an absolute requirement for this sequence.

DISCUSSION

Much of the regulation of human ADA gene expression occurs at the transcriptional level (7). In addition to regulation of transcriptional initiation, transcriptional pausing and arrest have been observed in the human and mouse ADA genes in tissues and cells with low levels of endogenous ADA (7,8,29). While the sequences responsible for the observed transcriptional arrest have been identified and characterized to some extent (18,30–33), the relative role of this mechanism *in vivo* in determining the tissue-specific pattern of ADA expression is not clear. The search for the regulatory regions in the human ADA gene responsible for activation of transcription initiation has revealed two, a proximal region upstream of the transcriptional start site which has promoter function and a region within the first intron with T-cell specific enhancer function. Initial characterization of the enhancer domain has been previously described (4,9). Examination of the promoter for potentially functional elements by sequence homology revealed six putative Sp1 binding sites and a poor homology TBP binding site (10,12). Somewhat similar elements have been identified in the mouse ADA gene promoter (21). An equivalent to the ADA first intron

enhancer has not been identified in mouse. However, distal sequences upstream of the mouse ADA promoter have been implicated in regulation of ADA expression in some tissues in transgenic mouse studies (34).

Role of potential TBP binding site

Initial examination of the human ADA promoter revealed a sequence TAAGAA, positioned at −21 to −26 bp, in the expected location for a TATA box. At first glance this sequence seems a plausible one to bind TBP, yet a weight matrix comparison of this sequence and the surrounding sequence to the TBP binding region of other eukaryotic promoters (22) indicated that it is a poor consensus match. Mutation of this sequence to one with no homology to the TATA box sequence, CTGCAG, resulted in only a slight decrease in promoter activation. This indicates that this particular sequence is not essential for promoter function and probably does not bind TBP strongly, although it may be loosely associated with it. Similar results have been reported for other genes including SV40 early genes (35) and *Xenopus* histone H2A (36). Deletion of the TBP binding site in these genes does not affect *in vivo* levels of expression. However, in both, the absence of this site allows for the increased usage of several of the minor start sites (36−38). It is possible that mutation of this sequence in the human ADA promoter has similar results. We were unable to map the transcription start sites for these TBP mutant clones directly to verify this hypothesis. By contrast, a similarly positioned sequence from the mouse ADA gene, TAAAAAA, is a better match to the TATA box consensus sequence. It has been shown to bind TFIID and to be required for transcription of the mouse ADA gene (21). This result is interesting in that it suggests that the murine ADA promoter and the human ADA promoter may function by slightly different mechanisms. This may in turn relate to some of the subtle variations in tissue expression of ADA between mice and humans.

The function of the TATA box in promoters which contain one is well studied. It is through TBP and some of its associated factors in the TFIID fraction commonly used for *in vitro* studies that both basal and activator-dependent transcription occur (39−43). The function of TBP in TATA-less promoters, of which the human ADA gene is an oft-cited example, is less well understood. TBP, as part of the TFIID complex, has been proven to be just as necessary for the function of these TATA-less promoters as for those with a TATA box (44). TBP is not the limiting factor in transcription from these promoters, implying that another protein(s) that interact with TBP are limiting (40). These other proteins, termed TBP associated factors or TAFs, are also necessary for transcription from TATA-less promoters. How TBP is directed to a TATA-less promoter is currently under study in many laboratories. Many divergent sequences have been shown to bind TBP with lower affinity (45,46). In some cases binding may represent a functional interaction between TBP and DNA in the absence of a specific recognition sequence (47) and changes in the binding site sequence may have only minor effects on this function. If such an array of divergent sequences are capable of recognizing and binding TBP, the question then arises what constitutes a TBP binding site? A recently proposed model, suggests that the context of the site *in vivo* may affect the binding of TBP to any given sequence (46). Binding of TBP to a weak binding site may be improved by the addition of favorable upstream or downstream sequences such as a site that binds an initiator protein or sites that bind activator proteins which are able to interact with TAFs. This model works admirably for the human ADA promoter which seems to possess at best a very weak TBP binding site. Mutation of this sequence did not significantly affect promoter activity, indicating that this sequence may have little direct involvement in recruiting TBP to this site. This function is performed by surrounding sequences such as the Sp1 binding sites or possibly sequences at the transcriptional start site which were not investigated. Since these sequences remained the same, TBP recruitment occured as usual and little loss of activation was observed.

Sp1 in basal promoter activation

The transcription factor Sp1 is required in both basal and enhanced activation of the ADA promoter. In the absence of binding sites for Sp1, no basal activation of the promoter is observed and enhancer-driven activation of the promoter is seriously compromised. All six of the Sp1 sites in the human ADA promoter have the ability to bind recombinant Sp1. Analysis by deletion/truncation of these Sp1 sites indicates that the distal sites VI and V are not necessary for basal transcription and the footprinting data suggests these sites may not bind Sp1 *in vivo*. Sp1 binds to the proximal sites I−IV synergistically to activate transcription much as described for other promoters both natural (24,48−54) and artificial (44,55−57). In general more proximal sites play a more important role than those distal to the start site and increasing the affinity of the most proximal site increases transcriptional activation (50). This result is not surprising since Sp1 has been shown to interact with TAF's (42−44,55,58). The ability of Sp1 to do this is probably the reason the human ADA gene can exist without an effective TBP binding site and yet maintain transcription. Basal activation of ADA should be highly dependent on the physiological concentration of Sp1 present in each tissue type. Low concentrations of Sp1 would result in the occupation of fewer sites and decreased synergistic activation of the ADA promoter. Sp1 protein and mRNA concentration in mouse tissues is highly variable (59). Some of the highest Sp1 expressing tissues in mouse, including columnar epithelial cells of the upper gastrointestinal tract, maternal placenta, and thymus, are also among the highest ADA expressing tissues in mouse (60−63). Most of the mouse tissues which express high levels of ADA also express high levels of Sp1. However, there are also tissues which express significant levels of Sp1 which express only low levels of ADA. Among these are lung and liver. Cells in these tissues may maintain lower levels of ADA transcription under high Sp1 concentration by utilization of other methods of transcriptional regulation, perhaps transcriptional arrest or pausing (7,8,29,33). Therefore, as we have found in transient asssay, Sp1 is necessary but not sufficient for high level ADA expression *in vivo*.

Promoter−enhancer interactions

The interactions between enhancer bound proteins and promoter bound ones are just beginning to be studied. Many roles for proximally bound Sp1 have been suggested and one or more of these may explain the enhancer requirement for such Sp1. The interaction of Sp1 with the TAF's may stabilize a component(s) of the transcriptional machinery (55). It is likely that the Sp1−TAF interaction is responsible for the positioning of TBP near the major transcription start site of the human ADA gene. Sp1−TAF interactions at each of the multiple Sp1 sites might also explain the multiple transcriptional start sites observed in this gene. This stabilization of the transcriptional machinery might then allow enhancer interactions with the assembled machinery

to occur more readily through different protein–protein interaction(s). There is evidence to support this idea. We have shown that the enhancer is able to activate transcription to low levels without proximal Sp1 sites present. This strongly suggests that at least some of the interactions of enhancer-bound proteins with the transcriptional machinery in the promoter region occurs independently of bound Sp1.

The favored model for enhancer–promoter interaction involves the association of bound factors at each site resulting in the looping out of the intervening DNA. This ability has recently been shown by Mastrangelo (64) and Su (65) for Sp1, whereby distantly bound Sp1 proteins interact to form multimers and loop out the intervening DNA. There is no doubt that the ADA promoter contains bound Sp1, but the enhancer in its smallest defined form does not contain a consensus Sp1 binding site (9). Sequences in the enhancer protected by methylation interference (ADA NF2) (9) are very similar but not identical to those reported by Kingsley (66) for another member of the Sp1 family, Sp2. The mechanism for ADA enhancer function may involve Sp1, or other members of its family, bound at or near the enhancer region interacting with bound Sp1 in the proximal promoter, but there is no direct evidence for this at the present.

It is unknown whether the enhancer requirement for Sp1 is specific or if it reflects a generic requirement for an activator. All other promoters tested previously in heterologous constructions with the CAT reporter gene and the ADA enhancer contained Sp1 sites in the promoter region (9). Analysis of enhancer activation of a promoter(s) lacking Sp1 sites but containing the binding sequence for other transcriptional activators should allow us to distinguish between the possibilities of enhancer interaction with only basal machinery or with both the machinery and Sp1. Studies at a detailed level await the identification, purification, and characterization of the relevant enhancer-binding proteins.

This initial characterization of the human ADA promoter gives us insight into how the promoter may be activated to low and moderate levels just by varying the levels of Sp1 in a tissue-specific manner. Preliminary studies of promoter–enhancer interactions have given us a glimpse of how other regulatory regions of the gene may interact with this basic unit to give high level activation in some tissues. More detailed characterization of the elements in the ADA enhancer and their relationship to each other will allow us to further examine the promoter–enhancer interaction.

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REFERENCES

1. Adams,A. and Harkness,R.A. (1976) *Clin. Exp. Immunol.*, **26**, 647–649.
2. Van der Weyden,M.B. and Kelley,W.N. (1976) *J. Biol. Chem.*, **251**, 5448–5456.
3. Hirschhorn,R., Martiniuk,F. and Rosen,F.S. (1978) *Clin. Immunol. Immunopath.*, **9**, 287–292.
4. Aronow,B., Lattier,D., Silbiger,R., Dusing,M., Hutton,J., Jones,G., Stock,J., McNeish,J., Potter,S., Witte,D. and Wiginton,D. (1989) *Genes Dev.*, **3**, 1384–1400.
5. Giblett,E.R., Anderson,J.E., Cohen,F., Pollara,B. and Meuwissen,H.J. (1972) *Lancet*, **2**, 1067–1069.
6. Martin,D.W. and Gelfand,E.W. (1981) *Ann. Rev. Biochem.*, **50**, 845–877.
7. Lattier,D.L., States,J.C., Hutton,J.J. and Wiginton, D.A. (1989) *Nucleic Acids Res.*, **17**, 1061–1076.
8. Chen,Z., Harless,M.L., Wright,D.A. and Kellems,R.E. (1990) *Mol. Cell. Biol.*, **10**, 4555–4564.
9. Aronow,B.J., Silbiger,R.N., Dusing,M.R., Stock,J.L., Yager,K.L., Potter,S.S., Hutton,J.J. and Wiginton,D.A. (1992) *Mol. Cell Biol.*, **12**, 4170–4185.
10. Valerio,D., Duyvesteyn,M.G.C., Dekker,B.M.M., Weeda,G., Berkvens,T.M., Van der Voorn,L., Van Ormondt,H. and Van der Eb,A.J. (1985) *EMBO J.*, **4**, 437–443.
11. Reitman,M., Lee,E., Westphal,H. and Felsenfeld,G. (1993) *Mol. Cell. Biol.*, **13**, 3990–3998.
12. Wiginton,D.A., Kaplan,D.J., States,J.C., Akeson,A.L., Perme,C.M., Bilyk,I.J., Vaughn,A.J., Lattier,D.L. and Hutton,J.J. (1986) *Biochem.*, **25**, 8234–8244.
13. Dynan, W. S. (1986) *Trends in Genet.*, **2**, 196–197.
14. Breathnach,R. and Chambon,P. (1981) *Annu. Rev. Biochem.*, **50**, 349–383.
15. Boshart,M., Kluppel,M., Schmidt,A., Schutz,G. and Luckow,B. (1992) *Gene*, **110**, 129–130.
16. Kadonaga,J.T., Carner,K.R., Masiarz,F.R. and Tjian,R. (1987) *Cell*, **51**, 1079–1090.
17. Ingolia,D.E., Al-Ubaidi,M.R., Yeung,C., Bigo,H.A., Wright,D.A. and Kellems,R.E. (1986) *Mol. Cell. Biol.*, **6**, 4458–4466.
18. Maa,M.-C., Chinsky,J.M., Ramamurthy,V., Martin,B.D. and Kellems,R.E. (1990) *J. Biol. Chem.*, **265**, 12513–12519.
19. Ackerman,S.L., Minden,A.G., Williams,G.T., Bobonis,C. and Yeung,C. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 7523–7527.
20. Al-Ubaidi,M.R., Ramamurthy,V., Maa,M., Ingolia,D.E., Chinsky,J.M., Martin,B.D. and Kellems,R.E. (1990) *Genomics*, **7**, 476–485.
21. Innis,J.W., Moore,D.J., Kash,S.F., Ramamurthy,V., Sawadogo,M. and Kellems,R.E. (1991) *J. Biol. Chem.*, **266**, 21765–21772.
22. Bucher,P. (1990) *J. Mol. Biol.*, **212**, 563–578.
23. Ciudad,C.J., Urlaub,G. and Chasin,L.A. (1988) *J. Biol. Chem.*, **263**, 16274–16282.
24. Blake,M.C., Jambou,R.C., Swick,A.G., Kahn,J.W. and Azizkhan,J.C. (1990) *Mol. Cell. Biol.*, **10**, 6632–6641.
25. Briggs,M.R., Kadonaga,J.T., Bell,S.P. and Tjian,R. (1986) *Science*, **234**, 47–52.
26. Kadonaga,J.T., Jones,K.A. and Tjian,R. (1986) *Trends Biochem. Sci.*, **11**, 20–23.
27. Kriwacki,R.W., Schultz,S.C., Steitz,T.A. and Caradonna,J.P. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 9759–9763.
28. Gorman,C., Moffat,L.F. and Howard,B.H. (1982) *Mol. Cell. Biol.*, **2**, 1044–1051
29. Chinsky,J.M., Maa,M.-C., Ramamurthy,V. and Kellems,R.E. (1989) *J. Biol. Chem.*, **264**, 14561–14565.
30. Chen,Z., Innis,J.W., Sun,M., Wright,D.A. and Kellems,R.E. (1991) *Mol. Cell. Biol.*, **11**, 6248–6256.
31. Ramamurthy,V., Maa,M.-C., Harless,M.L, Wright,D.A. and Kellems,R.E. (1990) *Mol. Cell. Biol.*, **10**, 1484–1491.
32. Innis,J.W. and Kellems,R.E. (1991) *Mol. Cell. Biol.*, **11**, 5398–5409.
33. Kash,S.F., Innis,J.W., Jackson,A.U. and Kellems,R.E. (1993) *Mol. Cell. Biol.*, **13**, 2718–2729.
34. Winston,J.H., Hanten,G.R., Overbeek,P.A. and Kellems,R.E. (1992) *J. Biol. Chem.*, **267**, 13472–13479.
35. Benoist,C. and Chambon,P. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 3865–3869.
36. Grosschedl,R. and Birnstiel,M.L. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 1432–1436.
37. Benoist,C. and Chambon,P. (1981) *Nature*, **290**, 304–310.
38. Mathis,D.J. and Chambon,P. (1981) *Nature*, **290**, 310–315.
39. Buratowski,S., Hahn,S., Guarente,L. and Sharp,P.A. (1989) *Cell*, **56**, 549–561.
40. Colgan,J. and Manley,J.L. (1992) *Genes Dev.*, **6**, 304–315.
41. Sawadogo,M. and Roeder,R.G. (1985) *Cell*, **43**, 165–175.
42. Gill,G. and Tjian,R. (1992) *Current Opinions in Genetics and Dev.*, **2**, 236–242.
43. Dynlacht,B.D., Hoey,T. and Tjian,R. (1991) *Cell*, **66**, 563–576.
44. Pugh,B.F. and Tjian,R. (1991) *Genes Dev.*, **5**, 1935–1945.

45. Hahn,S., Buratowski,S., Sharp,P.A. and Guarente,L. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 5718–5722.
46. Wiley,S.R., Kraus,R.J. and Mertz,J.E. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 5814–5818.
47. Zenzie-Gregory,B., Khachi,A., Garraway,I.P. and Smale,S.T. (1993) *Mol. Cell. Biol.*, **13**, 3841–3849.
48. Tasanen,K., Oikarinen,J., Kivirikko,K.I. and Pihlajaniemi,T. (1993) *Biochem. J.*, **292**, 41–45.
49. Pogulis,R.J. and Freytag,S.O. (1993) *J. Biol. Chem.*, **268**, 2493–2499.
50. Gidoni,D., Kadonaga,J.T., Barrera-Saldana,H., Takahashi,K., Chambon,P. and Tjian,R. (1985) *Science*, **230**, 511–517.
51. Proudfoot,N.J., Lee,B.A. and Monks,J. (1992) *New Biol.*, **4**, 369–381.
52. Chen,X., Azizkhan,J.C. and Lee,D.C. (1992) *Oncogene*, **7**, 1805–1815.
53. Jones,K.A., Yamamoto,K.R. and Tjian,R. (1985) *Cell*, **42**, 559–572.
54. Anderson,G.M. and Freytag,S.O. (1991) *Mol. Cell. Biol.*, **11**, 1935–1943.
55. Pugh,B.F. and Tjian,R. (1990) *Cell*, **61**, 1187–1197.
56. O'Shea-Greenfield,A. and Smale,S.T. (1992) *J. Biol. Chem.*, **267**, 1391–1402.
57. Smale,S.T. and Baltimore,D. (1989) *Cell*, **57**, 103–113.
58. Pugh,B.F. and Tjian,R. (1992) *J. Biol. Chem.*, **267**, 679–682.
59. Saffer,J.D., Jackson,S.P. and Annarella,M.B. (1991) *Mol. Cell. Biol.*, **11**, 2189–2199.
60. Brady,T.G. and O'Donovan,C.I. (1965) *Comp. Biochem. Physiol.*, **14**, 101–120.
61. Knudsen,T.B., Green,J.D., Airhart,M.J., Higley,H.R., Chinsky,J.M. and Kellems,R.E. (1988) *Biol. of Reprod.*, **39**, 937–951.
62. Chinsky,J.M., Ramamurthy,V., Fanslow,W.C., Ingolia,D.E., Blackburn,M.R., Shaffer,K.T., Higley,H.R., Trentin,J.J., Rudolph,F.B., Knudsen,T.B. and Kellems,R.E. (1990) *Differentiation*, **42**, 172–183.
63. Witte,D.P., Wiginton,D.A., Hutton,J.J. and Aronow,B.J. (1991) *J. Cell. Biol.*, **115**, 179–190.
64. Mastrangelo,I.A., Courey,A.J., Wall,J.S., Jackson,S.P. and Hough,P.V.C. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 5670–5674.
65. Su,W., Jackson,S., Tjian,R. and Echols,H. (1991) *Genes Dev.*, **5**, 820–826.
66. Kingsley,C. and Winoto,A. (1992) *Mol. Cell. Biol.*, **12**, 4251–4261.