Nucleic Acids Research, 1993, Vol. 21, No. 21 4873–4878

The Major Histocompatability Complex (MHC) Ea promoter: sequences and factors at the initiation site

R.Mantovani, L.Tora, V.Moncollin, J.M.Egly, C.Benoist* and D.Mathis
Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, Unité 184 de Biologie Moléculaire de Génie Génétique de l'INSERM, Institut de Chimie Biologique, Faculté de Médecine, 11 rue Humann, 67085 Strasbourg Cedex, France

Received August 10, 1993; Revised and Accepted September 16, 1993

ABSTRACT

We have analysed the function of sequences in the TATA/initiator region of the promoter of Ea, a class II gene of the Major Histocompatibility Complex. We find that the Ea promoter contains an initiator element with a strong influence on transcription. We also find that the Ea promoter does contain a bona fide TATA box, which can be recognized by the TATA binding protein (TBP), and that TBP is required for transcriptional activity. For activity, TBP must be included within a larger TFIID complex, as Ea transcription in a heat-treated extract can be restored by immunopurified TFIID but not by TBP alone. On the other hand, the TATA motif can be eliminated without significantly affecting either the efficiency or the startsites of transcription. This suggests that TBP, even in this TATA-containing promoter, is held in place by other components of the initiation complex, regardless of its affinity for the underlying DNA.

INTRODUCTION

Class II genes of the Major Histocompatibility Complex (MHC) encode heterodimeric membrane glycoproteins involved in a number of key immunological processes. They form a family of genes whose coordinated expression is elaborately regulated, being confined to a set of cells involved in the selection and activation of T cells (for reviews, see refs 1,2). The promoters of these genes, either murine or human, have been studied in detail by transgenesis, transfection into cultured cells and in vitro transcription, leading to the definition of two upstream elements of central importance—X and Y. The X box binds a number of transcription factors; these X-binding factors seem to have binding affinities and roles in transcriptional initiation which vary between class II genes (for review and refs, see 2). The Y box of all class II gene promoters, on the other hand, is recognized by the heteromeric CCAAT binding factor NF-Y. It contains a glutamine-rich activation domain, and is essential for the transcription of all the MHC class II genes tested (3,4). The promoter and enhancer activity of the X-Y motif is further modified by additional regulatory elements found immediately upstream of the X box in the S/W region, and by distant enhancer elements.

How do these promoter motifs operate to position the RNA polymerase in the preinitiation complex? To address this question we decided to investigate the function of sequence elements downstream of the X-Y motif, around the TATA box and the cap site.

Among the different elements which make up eukaryotic promoters for RNA polymerase II, the A/T-rich sequence known as the TATA box was the first to be recognized and is the most familiar (5). As the entry site for the general factor TFIID, which nucleates the formation of the preinitiation complex in vitro, the TATA box controls transcriptional initiation, both quantitatively and by positioning the startsites (for review, see 6). The factor thought to mediate the function of the TATA box, TFIID, is actually a multi-subunit complex. It contains a DNA-binding subunit, TBP, whose gene has recently been cloned from a number of species (for refs, see 6). TBP is associated with a number of other subunits, or TAFs (for TBP Associated Factors). While TBP does not vary between cell types, the TAFs may be heterogeneous in distinct forms of TFIID (7–11). The TFIID complex is thought to be the target of transcriptional activators or repressors (for review and discussion, see 6,12) and different TAFs may be associated with stimulation by different classes of activators (7–9). The central importance of TBP in eukaryotic transcription was underscored by the discovery that it is also essential for initiation by RNA polymerases I and III (for review and refs, see 13).

Given the pivotal role of the TATA box in the initiation of transcription by polymerase II, it is paradoxical that a number of genes do not contain recognizable TATA elements. These include a number of housekeeping genes, transcribed at slow and steady rates (reviewed in 14), but also tightly regulated genes such as that for terminal-deoxynucleotidyl-transferase, whose expression is strictly restricted to certain stages of lymphocyte ontogeny and differentiation (e.g. 15 and refs therein). The interpretation of these TATA-less promoters and of their mode of operation is currently a matter of debate. Do they directly bind TBP? Do they require TBP and TFIID at all? If so, how? (for reviews, see 6,16).

* To whom correspondence should be addressed at: LGME, Faculté de Médecine, 11 rue Humann, 67085 Strasbourg Cedex, France

Most of the promoters of MHC class II genes do contain at the expected position a TATA-like sequence that fits reasonably well with the consensus: GATTTTAAT in Ea, TTATTAT in Eb, TATTA in DRa, but the conservation in this region among the different MHC Class II promoters is nowhere near that of the X and Y motifs. We were prompted to study the role of TFIID in the transcription of MHC class II genes by an observation made during a linker-scanning analysis of the Ea promoter: a drastic 10 bp mutation which eliminated the TATA box affected neither the efficiency of transcription nor the position of the startsites (17). Similarly, the TATA box in the Aa promoter did not seem to play a strong quantitative role (18). These data suggested that the TATA box in MHC class II genes is dispensible and does not play its usual role in positioning initiation. It was even conceivable, in theory, that TBP would not be involved in Ea transcription.

To address these questions, we used recombinant TBP, immunopurified TFIID and a TBP-specific affinity-purified antibody. The data paint a somewhat unusual picture: the TATA box of the Ea promoter can bind TBP, and TBP is indeed essential for Ea transcription within a TFIID complex. Yet the affinity of its binding to DNA is not a major determinant of promoter efficacy. The TATA box does not appear to fix the transcriptional startsite, which rather results from the interplay between a strong initiator element and the X-Y complex.

MATERIALS AND METHODS

Recombinant proteins

The recombinant rTBPh and rTFIIB proteins were expressed in *E.coli* using the pET expression system based on the T7 promoter. The soluble proteins used in the footprinting assay were purified essentially to homogeneity by ion exchange and DNA-affinity chromotography, as described (19,20). The TFIIB-expressing plasmid was a generous gift from D. Reinberg (21).

rTBPh used for immunizations and affinity-purifications was prepared from inclusion bodies (80–90% pure TBP). These were solubilized in 6M Guanidinium HCl. Five volumes of NDB (100mM KCl, 20 mM Hepes ph 7.9, 0.5 mM EDTA, 1 mM DTT, 20% glycerol) were added; the diluted material was kept on ice for 10′ and then extensively dialyzed against 500 ml of NDB (6 hours, 2 buffer changes). To prepare columns for affinity-purification of antibodies this renatured rTBPh (1 mg in 2 ml) was coupled to 800 µl of CNBr-activated Sepharose (Pharmacia) according to the manufacturer's instructions.

Antibodies

A polyclonal mouse antiserum was raised by injecting mice with purified human recombinant TBP in CFA. Specific anti-TBP antibodies were purified from this serum as described (4).

The TBP column (see above) was washed with PBS and 1 ml of mouse serum (diluted to 10 ml with PBS) was applied three times; the column was then washed with 50 ml of PBS and the bound antibodies eluted with 15 ml of 0.1M glycine pH2.8 (1 ml fractions neutralized with 500 µl of 1M Tris–HCl pH8.0). Antibody-containing fractions were pooled, concentrated by Centricon centrifugation and dialyzed extensively against PBS. Antibody used in in vitro transcription experiments were further dialyzed against NDB.

The anti-TBP monoclonal antibody 3G3 has been described and was purified from ascites fluid as reported (7).

DNAase I footprinting

DNAse I footprinting consisted of a 30 min preincubation at 30°C in a 20 µl reaction volume containing ~1 ng (10000 cpm) of DNA probe, 100 ng of poly (dG-dC) (dG-dC), 0.05% NP-40, 5 mM $MgCl_2$ and 50 mg/ml BSA in buffer A (50 mM Tris–HCl pH 7.9, 0.1 mM EDTA, 0.5 mM DTT, 10% glycerol, 50 mM KCl). rTBP (40 ng; > 95% pure) and rTFIIB (20 ng; > 95% pure) were added as indicated. After the preincubation, 2 µl of DNAase I (10 mg/ml; Worthington, NJ) was added for 2 min at 30°C and reactions were stopped by adding 0.4 ml of a solution containing 0.5% SDS, 50 mM sodium acetate pH 5 and 50 mg/ml *E.coli* tRNA. DNA digestion products were analyzed on 8% acrylamide-8 M urea gels.

Western blot and *in vitro* transcription

Western blot and in vitro reactions with nuclear extracts from the murine B lymphoma CH27 and S1 analysis were performed exactly as detailed in refs 4 and 17. Heat inactivation of TFIID was achieved by incubating the extract at 46°C for 15 minutes.

TFIID immunoprecipitation and purification

The phenyl 5PW fraction (here refered to as ϕ; see Figure 4) was obtained as described (7). It was first dialysed against IP buffer [Tris–HCl 25 mM pH7.9, 5 mM $MgCl_2$, 10% glycerol, 0.1% NP40, 0.5 mM DTT, 0.1 M KCl]. To pre-clear the ϕ fraction it was incubated with protein G-Sepharose (Pharmacia) for 1.5 hours at 4°C. The anti-TBP antibody/protein G complexes were pelleted by centrifugation at 3000 rpm and then washed

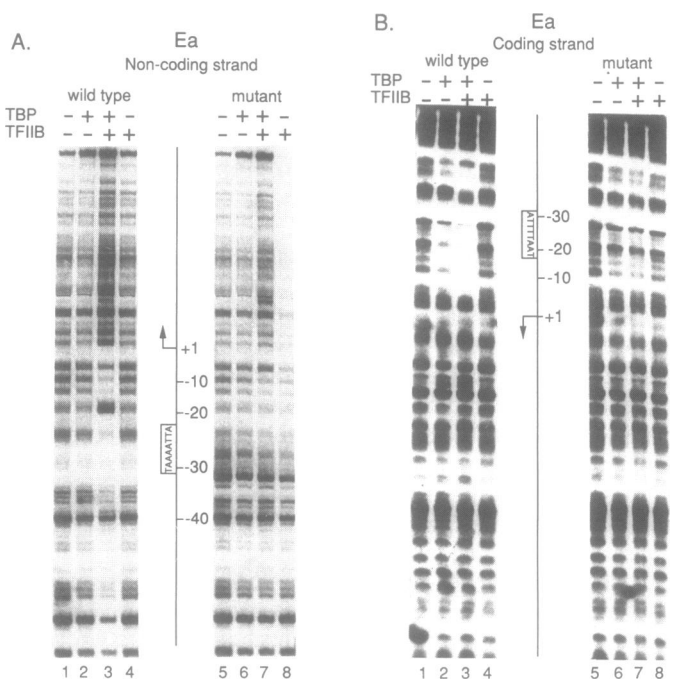

Figure 1. rTBP and rTFIIB bind the wild-type, but not the mutant, Ea TATA box. DNAse I footprint was performed as described in materials and methods. The DNA probes used encompassed the Ea promoter labelled on the non-coding (A) or coding (B) strand. The fragment was either wild-type (left) or carried the LS18 TATA box mutation (right). rTBPh and rTFIIB were added as indicated at the top of each panel. In each case, the location of the TATA-box and initiation site are indicated.

5 times with IP buffer. The linked antibody/protein G-Sepharose and the pre-cleared ϕ fraction were then mixed and incubated with rotation for 2 hours at 4°C. The complexes were subsequently pelleted and washed 5 times with NDB buffer as above. The first supernatant was conserved and tested in in vitro transcription reactions. The immuno-precipitated TFIID complexes were eluted by addition of an ~ 1000 fold excess of peptide PA81 for 3 hours at 4°C with rotation [this peptide is recognized by the binding site of the mAb 3G3 (22), and can thus compete against antibody/TBP binding]. After centrifugation at 3000 rpm the eluate was collected and tested by in vitro transcription. Routinely 2–5 ml of fraction ϕ were precipitated using 250 μl of protein G-sepharose and 400 μg of the purified antibody, and the TFIID was eluted in a 200–500 μl volume.

Gel retardation assays

1μl of the CH27 nuclear extract used for in vitro transcription was incubated with a double-stranded oligonucleotide (T-CTTGTTAATTCTGCCTCAGTCTGCGATCG) corresponding to sequences from positions −13 to +17 of the Ea promoter, in the following buffer: 50 mM NaCl, 10 mM Tris–HCl pH 7.5, 5% glycerol, 10 mM EDTA, 2 mM spermidine, 2 mM DTT, 300 ng poly(dI-dC). After incubation for 20 minutes at 25°C, samples were loaded on a 5% acrylamide gel in 0.5×TBE. We also used initiatior region oligonucleotides from the promoter of the Terminal-deoxynucleotidyl-transferase gene (TdT) (TCA-GAGCCCTCATTCTGGAGACACCAC; position −11/+16; ref 23) or the HIV promoter (TTGCCTGTACTGGGTCTCT-CTGGTTAGACC; position −12/+18; ref 24).

RESULTS AND DISCUSSION

TBP binds to the wild type but not a mutant Ea TATA box

Using 20 linker scanning mutants of the Ea promoter in transfections and in vitro transcription studies, we have identified the S, X and Y boxes as key regulatory elements (17). In this set of experiments we created the mutant LS18, carrying a drastic 10 base mutation in the TATA box (between positions −35 to −26; TTGGATTTTA to GGATCGATGC). Surprisingly, this template yielded wild type levels of expression upon transfection into MHC class II-expressing B cells or macrophages, and only a slight reduction of transcription in vitro (60% of wild-type activity); in both cases, transcription was initiated at the correct start site. Given the known loose DNA binding specificity of TBP (25,26), we decided to verify whether the Ea TATA box was indeed able to bind TBP and whether the mutant might still retain this in vitro binding capacity.

We used recombinant human TBP (rTBPh) purified as described and recently shown to bind efficiently to the TATA box of the Adenovirus major late promoter (AdML; 19). DNaseI footprinting experiments were performed with fragments of the wild type and LS18 mutant Ea promoters (positions −105 to +60). As shown in Figure 1, a weak protection over the TATA box appears on both strands when the wild-type DNA fragment is preincubated with rTBPh alone (Fig.1A, B lanes 1 and 2). With the AdML template, the addition of recombinant TFIIB (21) is known to stabilize the binding of TBP to the TATA box (20; VM, unpublished). Here also, addition of rTFIIB, which by itself does not bind (lanes 4), clearly enhances and modifies the footprint (lanes 3), which then extends from positions −35 to −5 relative to the Ea cap site; in addition, less prominent hypersensitive sites appear around position −40. On both strands the protection is centered on the TAAAATTA sequence. A region of weak protection also appears, located further upstream in an AT-rich stretch around position −60. In contrast, these footprints are essentially absent after parallel incubations of TBP and/or TFIIB with an Ea promoter fragment carrying the LS18 mutation in the TATA box (Figure 1A, B).

These results indicate that the Ea TATA box binds TBP and that this interaction is stabilized by the presence of TFIIB. These features are essentially lost in the LS18 TATA-box mutant, confirming that the mutation does prevent TBP binding in vitro, at least at the level of sensitivity provided by footprinting assays.

TBP is necessary for Ea transcription with wild type and LS 18 templates

Because of the normal transcriptional activity of the TATA mutant in spite of the apparent absence of TBP binding, we felt it important to investigate whether TBP was indeed required for Ea transcription. This was ascertained by transcription in vitro, with two lines of experimentation: we attempted to inhibit Ea transcription with affinity-purified antibodies to TBP; we also tested whether Ea transcription was heat-sensitive, a well-known characteristic of TBP and TFIID (27).

We used the in vitro transcription system consisting of the Ea promoter (−215 to +12) fused to a β-globin reporter gene (in the plasmid PE3; ref. 17) incubated with nuclear extracts from MHC class II$^+$ B cells of the CH27 lymphoma line; accurately initiated RNA was quantitated by S1-nuclease mapping. This transcription system has been shown to faithfully reproduce the in vivo situation, in that the start sites are accurate and transcription is fully dependent on the×and Y motifs (17,28). Anti NF-Y antibodies also have a profound inhibitory effect (4). We are thus dealing with an in vitro system that is dependent on upstream activators.

Antibodies against TBP were raised in mice immunized with recombinant TBP and purified over an affinity column containing

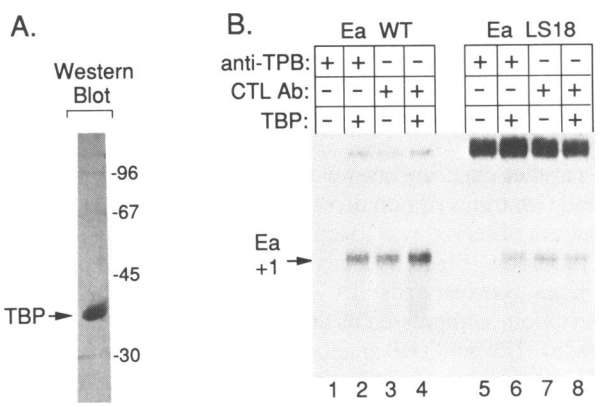

Figure 2. Anti-TBP antibodies inhibit Ea transcription in vitro. **A**: Western blot analysis of CH27 nuclear extract (200 μg) with affinity purified anti-TBP antibodies. The band corresponding to TBP has the expected molecular weight (38KD). **B**: Ea WT and Ea LS18 TATA-mutant template DNA were used for in vitro transcription assays using anti-TBP (lanes 1,2,5,6) or control antibodies (affinity purified rabbit anti–HEL; lanes 3,4,7,8). The antibodies (1 μg) were incubated with the extract for 15′ on ice, before addition of template DNA and nucleotides. In lanes 2, 4, 6 and 8 the inhibitory effect of the antibodies was blocked by preincubation with rTBPh (100 ng).

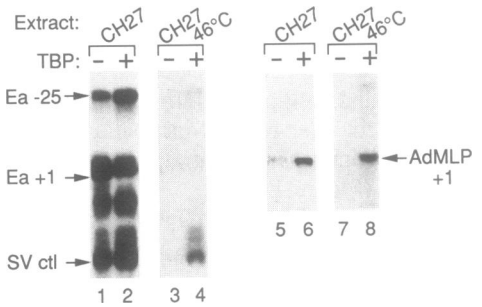

Figure 3. rTBPh restores SV40E and AdML, but not Ea transcription in a heat-treated CH27 nuclear extract. Normal or heat-treated (15' at 46°C) CH27 nuclear extracts were used for in vitro transcription driven by Ea and SV40E (lanes 1–4) or AdML (lanes 5–8) promoters. The internal control (SVCtl in lanes 1–4) consists of the SV40 enhancer and early promoter; the AdMLP contains just the TATA and Inr elements. All promoters are placed in front of a rabbit β-globin reporter gene (see ref 4); transcription was evaluated by S1 mapping, as above.

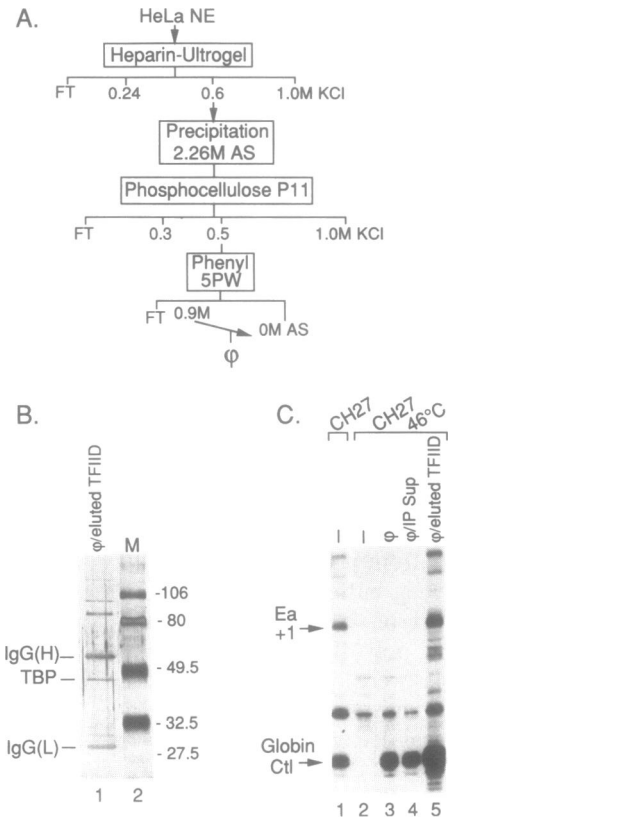

Figure 4. Purification of TBP-associated factor(s) necessary for Ea transcription. **A**: The general purification scheme, following TFIID activity through several chromatographic steps (for details see ref 7). **B**: an SDS-PAGE analysis of fraction φ, after immunoprecipitation with an anti-TBP monoclonal antibody and elution with the specific peptide. The band corresponding to TBP was identified by Western blotting (not shown). Some contamination is apparent from the immunoglobulin used for immunoprecipitation (IgGH and L). Panel **C** shows the ability of various fractions to functionally complement a heat-treated CH27 extract for Ea transcription in vitro. Lane 2: no addition: lane 3: fraction φ (see A); lane 4: the supernatant remaining after immunoprecipitation from fraction φ with anti TBP antibody; lane 5: TFIID released from anti-TBP immunoprecipitates by addition of an excess of peptide (see Methods). The globin ctl consists of the rabbit β-globin promoter (7) driving the same β-globin reporter segment as the Ea promoter. Transcription was evaluated by S1-mapping.

TBP linked to a Sepharose solid support, an essential step to obtain high affinity antibodies of narrow specificity. This approach also ensured that our preparation contained antibodies directed to different epitopes of TBP. The specificity of our purified anti-TBP preparation was verified by Western blotting with transcription-competent nuclear extracts: a single major band was detected, at the expected position for the 38 Kd TBP protein (Figure 2A).

Preincubation of the nuclear extract with the purified antibody, before the addition of template DNA and nucleotides, abolished transcription from the normal Ea start site at position +1 (Fig. 2B, lane 1). Control antibodies (affinity purified rabbit anti-lysozyme, see 4) had no effect on transcription (lane 3). As evidence for the specificity of the antibody inhibition, the effect could be blocked by preincubating the antibody with recombinant TBP prior to addition of extract, resulting in complete restoration of transcription (lane 2). Interestingly, and also confirming the specificity of the inhibition, the antibodies had no negative effect when added a few minutes after preincubation of the nuclear extract with template DNA (data not shown). This behaviour, which parallels observations with anti-NF-Y antibodies (4), indicates that the antibodies cannot affect a formed pre-initiation complex.

In parallel experiments we tested the effect of the anti-TBP antibody on transcription driven by the LS18 mutant promoter: the pattern observed was identical to that obtained with the wild type promoter (Fig. 2B, lanes 5–8; the heavy band on top of the lanes corresponds to TBP-independent read-through transcription, compressed at the position of the mutation by S1 cleavage). The anti-TBP antibody was also effective in inhibiting AdML promoter-driven transcription with CH27 nuclear or Hela whole cell extracts (RM, VM, data not shown).

These data demonstrate the TBP-dependance of both the wild type and TATA-mutant Ea promoters, ruling out the possibility that, because of the difference in TBP binding, they might act through different mechanisms.

To confirm these data, and to test how TBP might function in initiation of Ea trancription, we tested the efficiency of Ea driven templates in heat-treated nuclear extracts. TBP, and more generally TFIID, is quickly inactivated by incubation of extracts at moderate temperatures; several groups have shown that this loss of activity can be restored, for TATA-containing promoters, by the addition of recombinant TBP (for review and refs, see 6).

A 15 minute treatment of the CH27 nuclear extract at 46°C did indeed inactivate transcription from the Ea promoter, from the SV40 early promoter used here as an internal control, and from a minimal promoter containing just the AdML TATA box and initiator (Figure 3, lanes 3,7). Addition of recombinant TBP brought back substantial amounts of the SV40E and AdML transcripts, but not of the Ea transcripts initiated at position +1 (lanes 4,8). The lack of restoration of Ea transcription is not a simple quantitative effect, since high doses of rTBPh were used here (100ng per transcription reaction), and no effect was seen at even higher doses. On the other hand, an activity restoring Ea transcription in heat-treated extracts could be found to copurify with TFIID through several purification steps from crude nuclear extracts [Heparin-Ultrogel and Phosphocellulose (PC) columns; data not shown, Figure 4A]. We then used the TFIID immunopurification protocol recently established by some of us

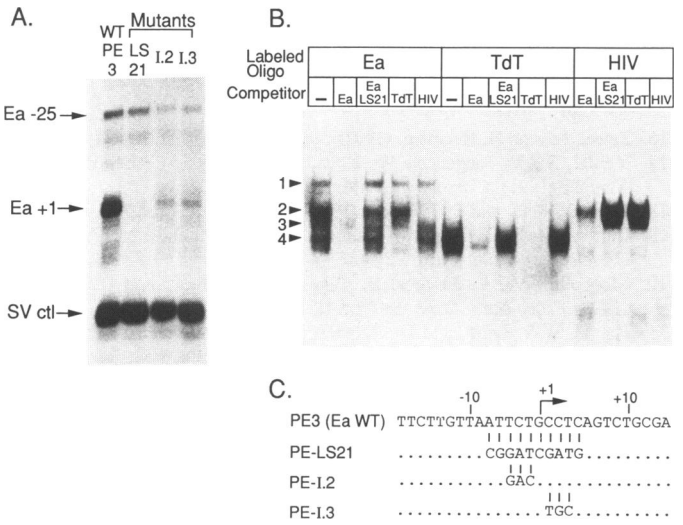

Figure 5. An initiator element is important for Ea promoter function. **A.** The CH27 in vitro transcription system was used with the WT plasmid PE3 and with the mutants PE-LS21, PE-I.2 and PE-I.3, whose sequences are shown in C. Transcription was evaluated by quantitative S1 mapping, as above. **B.** CH27 proteins binding to Ea initiator oligo are compared in electrophoretic mobility and cross-competition experiments with TdT and HIV initiator-binding factors. **C.** Mutations introduced in the PE mutant plasmids are shown together with the WT Ea sequence.

(7). In this protocol, TBP and its associated TAFs are immunoprecipitated with a monoclonal antibody (3G3), and can subsequently be released by an excess of a peptide which carries the antibody's epitope, namely the N-terminal 17 amino acids of TBP. As shown in Figure 4B, the fraction obtained by peptide elution after immunoprecipitation contains a discrete set of proteins as well as TBP (which can be identified by Western blotting; not shown).

Figure 4C presents the functional results of this fractionation scheme. To a limited degree, Ea transcription in the heat-treated CH27 extract can be restored by a partially purified fraction which contains TFIID (ϕ in Figure 4). The complementing activity is depleted in the supernatant remaining in this fraction after immunoprecipitation with the anti-TBP mAb (lane 4). In contrast, the peptide eluate obtained from the pellet after immunoprecipitating this fraction with the anti-TBP mAb is considerably enriched in complementing activity (lane 5). Interestingly, complementation is very much stronger than with the starting fraction, suggesting that an inhibitor might copurify with TFIID, but be removed by the immunoprecipitation procedure. The complementing activity in the immunoprecipitated pellet is, itself, heat-sensitive, being largely inactivated at 46°C (not shown). Here again, the LS18 mutant behaves like the wild-type promoter, and the same TAF-containing eluted TFIID fraction also restores its activity in a heat-treated extract (not shown).

Together with our previous results (17), the present data show a somewhat unusual behaviour of the Ea promoter.

On the one hand, it does contain a bona fide TATA box: the −32 to −24 motif fits the degenerate TATA consensus, and it binds TBP in what appears to be a relevant manner, since the binding is stabilized by TFIIB, as happens on a classical TATA-containing promoter such as AdML (Figure 1 and data not shown). TBP is also necessary for Ea transcription, which is specifically and completely inhibited by the anti-TBP antibody (Figure 2).

On the other hand, this TATA box is expendable for transcription in quantitative and qualitative terms, suggesting that Ea operates like a TATA-less promoter: the LS18 mutation does abolish the TBP binding detectable in the footprinting assay (Figure 1), but affects neither the efficiency of transcription nor the position of the startsite (17, and Figure 2). The LS18 mutant also behaves like the wild-type promoter, requiring for function TBP within a heat-sensitive TFIID complex which cannot be replaced by TBP alone (Figures 2, 3 and 4).

At this point, one cannot overlook the parallel between our data and those of Pugh and Tjian (31), using an artificial TATA-less promoter: in both cases, TBP binding to DNA, assayed by footprinting, is absent or unimportant, yet TBP itself is critical; transcription is dependent on a dominant upstream activator together with an initiator; this activator contains glutamine-rich activation domains; and transcription requires a thermolabile TFIID complex, other than TBP, which is not as essential for TATA-containing promoters. Pugh and Tjian (31) have proposed that TBP does not bind DNA in TATA-less promoters, but is held in place by a 'tethering factor'. Based on this analogy, one would propose that, for the Ea promoter, the primary force which holds TBP in place is not its affinity for the Ea TATA box, but rather protein/protein interactions with other factors. Here also, the requirement for a TAF to anchor TBP might account for the fact that isolated TBP is strictly incapable of restoring Ea transcription in a heat-treated extract. Whether this TAF is itself heat-sensitive, or simply cannot exchange from an inactivated TFIID complex onto fresh TBP, remains an open question, as in Pugh and Tjian's experiments.

Our results are compatible with the mutagenesis study of Dedrick and Jones (18), but appear somewhat at odds with those of Matsushima et al. (32), which implied an important role of the TATA box for transcription of DRa, the human homolog of Ea. In that study, though, an estimation of the amounts of correctly initiated RNAs was hampered by the low resolution and the apparent high variability of the in vitro analysis. Otherwise, it is quite possible that the balance of initiation factors is different in DRa compared to other MHC class II genes, due to the presence of an octamer binding site between the Y box and the TATA box, a site which appears unique to DRa.

The Ea promoter contains a strong initiator motif

If the TATA box is dispensible for the selection of transcriptional startsites, how is initiation positioned? Initiator motifs located immediately at the cap site are known to perform this positioning function in several TATA-less promoters (e.g. 23,29,30). The existence of an initiator element in the Ea promoter was made all the more plausible by the existence of sequence similarity between sequences at the Ea capsite and the TdT initiator motif (GCCTCAGTCTGC and CCCTCATTCTGG). We thus introduced several mutations in this region of our PE3 plasmid (sequences shown in Figure 5C), and tested these mutant templates in the in vitro transcription assay (Figure 5A). The ten base replacement in the PE-LS21 mutant led to a severe drop in Ea transcription (to 5% ±2 of WT transcriptional efficiency in four independent experiments), while the PE-I.2 and PE-I.3 were crippled to a lesser, but still significant degree (to 33% ±4 and 30% ±5, respectively). Note that in all the mutants only

the correct Ea +1 signal was influenced, whereas the spurious −25 transcript remained largely unchanged.

To further substantiate the existence of an initiator element in Ea, we compared the factor-binding potential of this region with that of other known initiator motifs. A ds-oligonucleotide covering the Ea initiator region was used in parallel and in cross-competition with oligonucleotides of similar sizes containing know initiator motifs from the TdT and HIV promoters (23,24). As shown in Figure 5B, the binding pattern observed with the Ea oligo is complex: four diferent bands are observed; they appear specific in that all are eliminated by competition with an excess of cold WT Ea oligo, but not by an oligo containing the drastic LS21 mutation.

Interestingly, one of the bands (band 4) roughly comigrates with a retarded band observed with the TdT oligo. That the Ea and TdT motifs bind the same factor is further substantiated by the efficient cross-competition for binding at this position. In contrast, the other retarded bands observed with the Ea oligo are not competed off by the TdT oligo.

One of these, band 2, appears shared between the Ea and HIV templates. Band 2 seen with the Ea template comigrates with the major retarded band found with the HIV template, and there is efficient cross-competition between the templates. This band might correspond to the LBP1 factor described by Kato et al (24), as it is abolished by mutations in the HIV oligo which inhibit LBP1 binding (data not shown). Note that band 2 is unrelated to band 4 (and the major band seen with the TdT oligo), as there is no cross-competition between TdT and HIV oligos.

In summary, the Ea promoter contains a strong initiator element of profound functional importance. It binds a complex set of proteins, related in part to several independent factors which bind initiator motifs in other genes. This complexity will need to be unravelled in further studies.

CONCLUSIONS

What, then, does the choice of the initiation sites in Ea actually depend on? Previous analyses with transgenic mice strongly implicated the upstream X and Y boxes (33; Viville et al., unpublished). Our present data also point to the influence of an initiator element at the cap site. One is left with the notion that the position of the initiation site in Ea depends on the conjunction of the strong promoter/enhancer elements at the X-Y motif with the initiator element which serves to focus transcription.

REFERENCES

1. Benoist, C., and D. Mathis. (1990) *Annu. Rev. Immunol.*, **8**, 681−715.
2. Glimcher, L.H., and C.J. Kara. (1992) *Annu. Rev. Immunol.*, **10**, 13−49.
3. Li, X.Y., R. Hooft van Huijsduijnen, R. Mantovani, C. Benoist, and D. Mathis. (1992) *J. Biol. Chem.*, **267**, 8984−8990.
4. Mantovani, R., U. Pessara, F. Tronche, X-Y. Li, A-M. Knapp, J.L. Pasquali, C. Benoist, D. Mathis. (1992) *EMBO J.*, **11**, 3315−3322.
5. Breathnach, R., and P. Chambon. (1981) *Ann. Rev. Biochem.*, **50**, 349−403.
6. Pugh, B.F., and R. Tjian. (1992) *J. Biol. Chem.* **267**, 679−682.
7. Brou, C., J. Wu, S. Ali, E. Scheer, C. Lang, I. Davidson, P. Chambon, L. Tora. (1993) *Nucl. Ac. Res.*, **21**, 5−12
8. Dynlacht, B.D., T. Hoey, and R. Tjian. (1991) *Cell*, **66**, 563−575.
9. Tanese, N., B.F. Pugh, and R. Tjian. (1991) *Genes and Development*, **5**, 2212−2224.
10. Timmers, H.T.M., and P.A. Sharp. (1991) *Genes and Development*, **5**, 1946−1956.
11. Weinzierl, R.O.J., B.D. Dynlacht and R. Tjian. (1993) *Nature*, **362**, 511−516.
12. Greenblatt, J. (1991) *Cell*, **66**, 1067−1070.
13. Sharp, P.A. (1992) *Cell*, **68**, 819−821.
14. Dynan, J. (1986) *T.I.G.*, **2**, 196−197.
15. Bogue, M., S. Gilfillan, C. Benoist, D. Mathis. (1992) *Proc. Natl. Acad. Sci.*, **89**, 11011−11015.
16. Zawel, L., and D. Reinberg. (1992) *Current Opinion Cell Biol.*, **4**, 488−495.
17. Viville, S., V. Jongeneel, W. Koch, R. Mantovani, C. Benoist, and D. Mathis. (1991) *J. Immunol.*, **146**, 3211−3217
18. Dedrick, R.L. and P.P. Jones. (1990) *Mol. & Cell. Biol.*, **10**, 593−604.
19. Burton, N., B. Cavallini, M. Kanno, V. Moncollin, and J.M. Egly. (1991) *Protein Expression Purif.*, **2**, 432−441.
20. Moncollin, V., L. Fischer, B. Cavallini, J-M. Egly, and P. Chambon. (1991).*Proc. Natl. Acad. Sci. USA.*, **89**, 397−401.
21. Ha, I., W.S. Lane, and D. Reinberg. (1992) *Nature*, **352**, 689−695.
22. Brou, C., S. Chaudhary, I. Davidson, Y. Lutz, J. Wu, J.M. Egly, L. Tora, and P. Chambon. (1993) *EMBO J.*, **12**, 489−499.
23. Smale, S.T., M.C. Schmidt, A.J. Berk, and D. Baltimore. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 4509−4513.
24. Kato H., M. Horikoshi R.G. Roeder. (1991) *Science* **251**, 1476−1479.
25. Hahn, S., S. Buratowski, P.A. Sharp, and L Guarente. (1989) *Proc. Natl. Acad. Sci. USA.*, **86**, 5718−5722.
26. Singer, V.L., C.R. Wobbe, and K. Struhl. (1990) *Genes and Development*, **4**, 636−645.
27. Nakajima, N., M. Horikoshi, and R.G. Roeder. (1988) *Mol. Cell. Biol.*, **8**, 4028−4040.
28. Kouskoff, V., R.M. Mantovani, S.M. Candeias, A. Dorn, A. Staub, B. Liswoska-Grospierre, C. Griscelli, C. Benoist, and D. Mathis. (1991) *J. Immunol.*, **146**, 3197−3204.
29. Carcamo, J., L. Buckbinder, and D. Reinberg. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 8052−8056.
30. Zenzie-Gregory, B. A. O'Shea-Greenfield, and S.T. Smale. (1992) *J. Biol. Chem.*, **267**, 2823−2830.
31. Pugh, B.F., and R. Tjian. (1991) *Genes and Development*, **5**, 1935−1945.
32. Matsushima G., Y. Itoh-Lindstrom, and J. Ting. (1992) *Mol. Cell. Biol.* **12**, 5610−5619.
33. Dorn, A., B. Durand, C. Marfing, M. Lemeur, C. Benoist, and D. Mathis. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 6249−6253.