MOLECULAR AND CELLULAR BIOLOGY, Feb. 1996, p. 503–512 Vol. 16, No. 2
0270-7306/96/$04.00+0

The Major Histocompatibility Complex Class II Ea Promoter Requires TFIID Binding to an Initiator Sequence

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Received 12 July 1995/Returned for modification 22 August 1995/Accepted 10 November 1995

The major histocompatibility complex (MHC) class II Ea promoter is dependent on the presence of conserved upstream X and Y boxes and of initiator (Inr) sequences. In vitro transcription analysis of the Inr region with linker-scanning mutants pinpoints a functionally essential element that shows homology to the terminal deoxynucleotidyltransferase (TdT) Inr; contrary to the TdT Inr and other Inrs identified so far, the key sequence, between positions +5 and +12, is located within a transcribed area. Swapping the TdT sequence into the corresponding Ea position leads to a fivefold increase in transcription rate, without altering start site selection. Inr-binding proteins LBP-1/CP2 and TIP—a TdT Inr-binding protein unrelated to YY1—recognize the Ea Inr; they interact with overlapping yet distinct sequences around the Cap site, but their binding does not coincide with Ea Inr activity. A good correlation is, rather, found with binding of immunopurified holo-TFIID to this element. TFIID interacts both with Ea TATA-like and Inr sequences, but only the latter is functionally relevant. Unlike TBP, TFIID binds in the absence of TFIIA, indicating a stabilizing role for TBP-associated factors in Ea promoter recognition. Sequence comparison with other mouse and human MHC class II promoters suggests a common mechanism of start site(s) selection for the MHC class II gene family.

Major histocompatibility complex (MHC) class II molecules are heterodimeric proteins known to play a key role in several immunological functions (reference 8 and references therein). Both in humans and mice they are encoded by several genes whose expression is controlled at the transcriptional level in a tissue-specific and developmentally regulated manner, by means of their promoter sequences (see references 3 and 17 for reviews). Functional analysis of all MHC class II promoters established that they share common regulatory elements: the highly conserved Y, X, and X2 boxes and an additional pyrimidine stretch—the S box—upstream of X. They bind activators such as NF-Y (12, 34), RF-X/NF-X (30, 46), and members of the AP1 or ATF families, respectively (1, 23). Interestingly, conservation is not extended to the TATA box: comparative analysis of such promoters failed to identify bona fide TATA boxes at the usual −25 to −30 position, even though in some cases AT-rich sequences are found (3). Conflicting results concerning the role of the AT-rich regions at position −30 have been obtained: Aa and Ea can be considered functionally TATA-less (10, 56), but in the Dra promoter (the human homolog of Ea), removal of this stretch led to altered start site selection patterns (37).

The TATA box is the most common regulatory element in polymerase II promoters (7) and plays a key role in regulating the overall level of transcription and in selecting the start sites (5); it is recognized by TBP, a DNA-binding protein whose genes have been cloned from different species. Biochemical and in vitro transcription studies indicate that TBP is tightly associated with several other polypeptides (TBP-associated factors [TAFs]) as part of the multisubunit TFIID complex (53), which nucleates the initial events in the ordered assembly of the preinitiation complex, involving other general transcription factors (63).

In promoters that lack the TATA box, sequences surrounding the transcription start site, the initiator (Inr), play an essential role (see reference 58 for a review); thorough mutagenesis studies performed on the terminal deoxynucleotidyltransferase (TdT) Inr, one of the Inrs which is linked to a TATA-less promoter, identified a sequence (26, 41, 51) that is very similar to the loose consensus found by Bucher to be present around initiation start sites, on the basis of a computer-based search for conserved regulatory elements in polymerase II promoters (7). In the search for *trans*-acting factors responsible for the activity of this *cis*-acting element, several Inr-binding proteins recognizing different Inrs have been described: HIP1/E2F, binding to DHFR Inr (38); YY1, binding to TdT and P5 Inrs (49); TFII-I, involved in adenovirus major late (AdML) Inr activity (48); USF, which stimulates AdML and human immunodeficiency virus (HIV) Inrs (13); LBP-1, regulating the HIV Inr (27); and MYC/MAX, binding to the λ5 Inr (33). Despite the functional importance of such proteins for Inr activity in different systems, recent results failed to implicate YY1 in transcriptional activation of the TdT Inr (26); rather, a good correlation between binding of the TFIID complex to TdT Inr sequences and function was found (28), a result in line with another study demonstrating that highly purified TFIID directs TdT Inr stimulation irrespective of activation from distal regulatory elements and that the TATA-binding activity is not required for the initial recruitment to the TdT Inr (36). Other mechanisms have been postulated to be involved in transcriptional activation by Inrs: weak binding of TBP to nonconsensus sequences at −30 has been suggested to be necessary for Inr activity (59, 64), and a heat-labile tethering factor in TFIID was shown to be responsible for mediating Sp1-Inr connections in a TATA-less promoter (44). Functional indications that there are different classes of Inrs have been

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TABLE 1. List of oligonucleotides used in EMSA[a]

Oligonucleotide	Sequence
HIVI	TTGCCTGTACTGGGTCTCTCGGTTAGACC
HIVI Mut	TTGCCTGTAGATGGTCTCTAGTTAGACC
TdTI	AGAGCCCTCATTCTGGAGACACCAC
TdTI Mut	AGAGCCCTGGGTCTGGAGACACCAC
DraI	TTCTTTTATTCTTGTCTGTTCTGCCTCACT
P5+1	CGGGAGGGTCTCCATTTTGAAGCGG
P5-60	GTTTGCGACATTTTGCGACAC
LeII	CCCTAGGTTTTCTGGAGACTGA
MLI	GCGTTCGTCCTCACTCTCTTCCGCATCA
TeF1I	TTCCTGTCCTCATCCGAACATTCTT
E2F	GGCTCTTTCGCGGCAAAAAGG
λ5I	TACACAGATCCACCTGCACTGGAA
EaI	TCTTGTTAATTCTGCCTCAGTCTGCGATCG
EaI1	TCTTGTTCCGTCTGCCTCAGTCTGCGATCG
EaI2	TCTTGTTAATGACGCCTCAGTCTGCGATCG
EaI3	TCTTGTTAATTCTGTGCCAGTCTGCGATCG
EaI4	TCTTGTTAATTCTGCCTGTCTCTGCGATCG
EaI5	TCTTGTTAATTCTGCCTCAGAGGGCGATCG
EaI6	TCTTGTTAATTCTGCCTCAGTCTCGAATCG
EaI7	TCTTGTTAATTCTGCCTCAGTCTAAAATCG
EaI8	TCTTGTTAATTCTTCCTCAGTCTGCGATCG
EaLS21	TCTTGTTACGGCTCGATGAGTCTGCGATCG
ML TATA	GAAGGGGGGTATAAAAGGGGGTGGGGGCGTTCGTCCT
Ea TATA	TGCTTTGGATTTTAATCCCTTTT
Sp1	GATCCCCCGCCCC

[a] Underlined bases represent mutations with respect to the wild-type sequences.

obtained (65), a fact that helps reconcile the apparent discrepancies so far observed.

In the case of Ea, we have demonstrated the following. (i) The TATA box, despite normal TBP binding, is functionally irrelevant, both in vivo and in vitro (56). (ii) TBP cannot restore the basal level of heat-inactivated transcription. (iii) Purification of a heat-labile factor necessary for Ea transcription followed TFIID activity, and it was found in one (or more) of the TAFs present in a highly purified TFIID fraction (35). (iv) The sequences around the major start site are important for promoter function and bind proteins that cross-compete with HIV and TdT Inrs (35).

Starting from these observations, we wished to extend our understanding of the mechanisms of action of the Ea Inr: it seemed important to know to which class of Inrs it would belong, which proteins bind to it, and what their roles are; finally, we wanted to verify whether TFIID could influence Inr activity. To answer these questions, we used in vitro transcription and electrophoretic mobility shift assay (EMSA) with Ea Inr mutants.

MATERIALS AND METHODS

EMSA. EMSA of Inr-binding proteins was performed as previously described (35). EMSA of TFIID in agarose gels was done as follows. Immunopurified TFIID fractions (0.3 μl), with or without pure TFIIA, were incubated in TBP buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.9], 50 mM NaCl, 8% glycerol, 2% PEG 6000, 5 mM ammonium sulfate, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol) together with 10,000 cpm of ^{32}P-labelled TATA-containing AdML oligonucleotide (−38 to −4 [Table 1]) or Ea fragments; the total volume was 10 μl. After incubation for 45 min at 30°C, we added 2 μl of 1× TBP buffer containing bromophenol blue, and samples were loaded on a 1.5% agarose gel (Bio-Rad Ultrapure) in 0.5× Tris-borate-EDTA. Gels were run at 140 V for 90 min, transferred to DE81 paper, vacuum-dried, and exposed.

The Ea fragments used in Fig. 5 and 6 were obtained by PCR and contained sequences from −74 to +16 of the Ea promoter, either wild type or with a mutation in the TATA box (35, 56) or in the Inr. To ensure that all fragments had the same specific activity and thus that binding affinities could be quantitatively comparable, we labelled the common 5′ oligonucleotide, corresponding to Y39mer (12), and used it for PCRs with the different Inr mutant oligonucleotides listed in Table 1.

Antibodies and supershift EMSA. For supershift experiments, anti-LBP-1 antibodies (62) were purified on antigen columns according to the protocol described in reference 34. Antigen columns were prepared by linking recombinant *Escherichia coli*-made LBP-1a (500 μg) from inclusion bodies to a CnBr-activated Sepharose column (Pharmacia) according to the manufacturer's instructions. Anti-$hTAF_{II}100$ 1TA (24) was purified by precipitation with ammonium sulfate, resuspension in phosphate-buffered saline, and dialysis against NDB (20% glycerol, 20 mM HEPES [pH 7.9], 100 mM KCl, 0.2 mM EDTA, 1 mM dithiothreitol). Supershift experiments were performed by preincubating TFIID with 20, 100, and 500 ng of purified anti-$hTAF_{II}100$ or 500 ng of purified anti-NF-YA (34) antibodies for 2 h on ice, before addition of the labelled DNA.

Purification of TFIID-TFIIA and production of recombinant LBP-1 and TBP. Production and purification of recombinant His-tagged LBP-1a, LBP-1b, and LBP-1c from *E. coli* were performed as previously described (62); holo-TFIID from HeLa cells were immunopurified with an anti-TBP monoclonal antibody (3G3), according to the method described in references 6 and 24. Three independent preparations of purified TFIID were used in EMSA. TFIIA was purified from HeLa cells by following the protocol described in reference 11, except that the nuclear extract was first passed on heparin-agarose and the flowthrough fraction was collected and loaded directly on an Ni-agarose resin (Qiagen). Production of recombinant TBP was previously described (35).

Construction of Ea Inr mutants. The Ea Inr mutants were constructed by PCR by using 3′ oligonucleotides harboring the desired mutations and amplifying the Ea promoter-containing PX3 template (−215 to +12), together with a 5′ oligonucleotide in the −215 region (30); the 5′ and 3′ oligonucleotides had *Xho*I and *Bam*HI sites, respectively, in their flanking sequences; after PCR amplification, the fragments were cut with the two enzymes and cloned in the PA101 plasmid (50). The simian virus 40 (SV40) promoter was then removed by cutting with *Bam*HI and *Hin*dIII, filling in with the Klenow fragment, and religating (56). The PEm mutants were generated by oligonucleotide mutagenesis in M13 and transferred into the SV40 promoter-containing PX3 plasmid; subsequently, the SV40 promoter was excised with *Bam*HI and *Hin*dIII. All plasmids were checked by sequencing.

In vitro transcription. The CH27 Ea in vitro transcription and S1 mapping were performed as detailed in references 30 and 56. Since mutations occur around the start site region, to avoid S1 cutting in the mismatch region that would occur with a wild-type Ea probe, we prepared a different probe for every mutant: the single-stranded probe was obtained with double-stranded plasmids containing the individual mutation, linearized with *Eco*RI according to the method described in reference 57. To make sure that all probes had the same specific activity, the M6 (30, 56, 57) primer used for preparation (60 pmol) was ^{32}P labelled with polynucleotide kinase, and then 5 pmol of this labelling reaction mixture was used to make individual probes for the different mutants. At least two independent plasmid preparations of all mutants were tested in three separate experiments.

RESULTS

Functional in vitro dissection of the Ea Inr. In a previous study we showed that the Ea promoter contains functionally important sequences around the start site (35); we initially wished to better define the nucleotides necessary for this activity. For this purpose, we used an in vitro transcription system consisting of the Ea promoter (−215 to +12) fused to a β-globin reporter gene, which is faithfully transcribed by MHC class II-positive B-cell CH27 nuclear extracts; to help normalize the values, we added to the reaction mixtures an SV40 promoter-driven internal control. S1 analysis of transcripts allows the evaluation of quantitative and qualitative changes in the transcription rate. This system is absolutely dependent on upstream X, X2, and Y boxes (30, 56). We introduced linker-scanning mutants of 3 bp, from position −6, the limit of the functionally irrelevant region (56), to +13, the end of the Ea promoter sequences (detailed in Fig. 1A). To test whether the Ea start site region is indeed an Inr, we also exchanged the 10-bp LS21 mutant with sequences of the TdT Inr constituting the minimal core Inr (positions −5 to +5 [26, 51]). Analysis of the different mutants (Fig. 1B) strongly indicates that I4, I5, and I6 have extremely reduced (by 10-fold) transcription rates; I1 and I8 behave essentially like the wild type, while I2, I3, and I7 have intermediate levels. Interestingly, I9, which contains the TdT core Inr placed exactly in the corresponding Ea re-

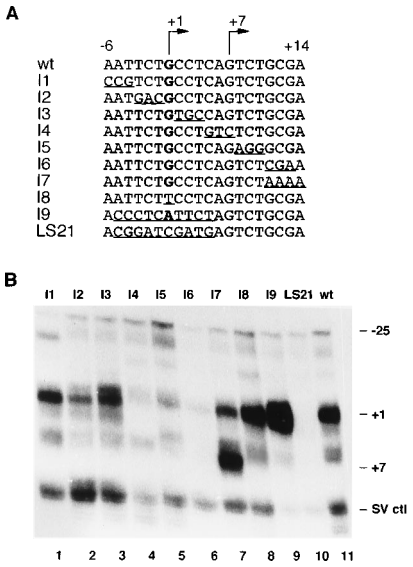


FIG. 1. In vitro transcription analysis of Ea Inr mutants. (A) The different mutants are depicted, with the major start site used in vivo and in vitro indicated in boldface type. Introduced mutations are underlined. (B) The normal Ea +1 signal as well as the alternative +7 signal is indicated. The spurious −25 signal, refractory to mutations, and the shorter signal generated by the SV40 control, are used as internal standards. wt, wild type.

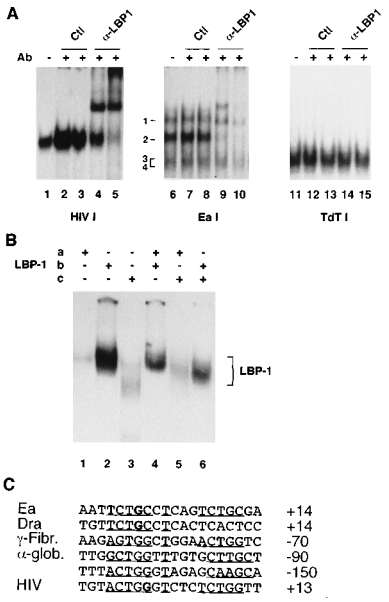


FIG. 2. Band 2 is LBP-1/CP2. (A) EMSA of band 2 with control (lanes 2, 3, 7, 8, 12, and 13) and anti-LBP-1 antibodies (lanes 4, 5, 9, 10, 14, and 15) with HIV Inr (lanes 1 to 5), Ea Inr (lanes 6 to 10) and TdT Inr (lanes 11 to 15). We preincubated 5 μg of CH27 nuclear extracts with the antibodies for 15 min on ice before addition of the labelled oligonucleotide. (B) EMSA with Ea Inr oligonucleotide and recombinant LBP-1a, LBP-1b, or LBP-1c alone (lanes 1 to 3, respectively) or in different combinations (as indicated; lanes 4 to 6). (C) Known LBP-1/CP2 binding sites are listed. The two a/tCTGg/c repeats are underlined. Start sites are in boldface type.

gion, induces a strong increase in transcription (fivefold over that of the wild-type Ea), which is particularly striking compared with the scrambled sequence of EaLS21 (Fig. 1B, compare lanes 9 to 11); this quantitative change is focused on the same major start site that is normally used by Ea. On the other hand, mutant I7, while decreasing the level of the major +1 start site, shows the appearance of an additional start site at +7. The two latter results, restoration of activity by TdT core Inr and modification of start site selection, strongly indicate that the Ea start site region does contain a classical Inr and that the core of this activity is positioned between positions +5 and +12 (I4 to I6), with additional minor effects of the −3 to +4 area.

EMSA analysis of Ea Inr-binding proteins. By performing EMSA with an Ea oligonucleotide containing the functionally relevant sequences, we previously detected several bands (designated 1 to 4) resulting from specific interactions between DNA-binding proteins and the Ea −13 to +17 region (35). Some of these proteins (band 3-4) were competed for by a TdT Inr oligonucleotide, while another (band 2) was competed for by an HIV Inr oligonucleotide, suggesting that Inr-binding proteins recognize such regions. It has been reported that the cross-competing HIV oligonucleotide binds with high affinity the LBP-1/CP2 proteins (27, 43, 62), whose genes were identified in different species, including *Drosophila melanogaster* (4, 14, 32, 62). We decided to verify whether band 2 indeed corresponds to LBP-1, by taking advantage of the anti-LBP-1 rabbit antiserum described for EMSA supershift experiments (62). Figure 2A shows that band 2 binding to the Ea Inr and HIV Inr oligonucleotides is supershifted by the addition of increasing amounts of the purified antibodies (lanes 1, 4, 5, 6, 9, and 10), while band 3-4 of either the Ea Inr or the TdT Inr oligonucleotide is left unchanged (lanes 6, 9, 10, 11, 14, and 15). Control preimmune antiserum does not modify the binding pattern of any of the three oligonucleotides used (lanes 1 to 3, 6 to 8, and 11 to 13). As further support for the idea that LBP-1 binds the Ea Inr oligonucleotide, we expressed and purified recombinant *E. coli*-made His-tagged LBP-1a, LBP-1b, and LBP-1c (62) and used them in EMSA. The three isoforms of LBP-1 induce complexes of different mobilities (Fig. 2B, lanes 1 to 3); such complexes are specific, being abolished by an excess of cold oligonucleotide and supershifted by the specific antibodies (data not shown). Since the three proteins are known to bind DNA as dimers and are able to form heterodimers between them, we mixed the different LBP-1/CP2 isoforms and observed a change in their electrophoretic mobilities, with the formation of intermediate complexes (Fig. 2B, compare lanes 1, 2, and 4; lanes 1, 3, and 5; and lanes 2, 3, and 6). Thus, we conclude that the Ea initiator region contains an LBP-1 binding site which binds homo- and heterodimers of the different LBP-1/CP2 isoforms.

We next tried to identify band 3-4 (we shall call this activity TIP, for TdT Inr protein) by doing competition experiments with several Inr-containing oligonucleotides known to bind well-identified Inr-binding proteins (Table 1). Results of such experiments are shown in Fig. 3A: as expected, self competition abolished all four bands, while the addition of HIV Inr (but not of the HIV Inr mutant) and TdT Inr (but not the TdT Inr mutant) abolished LBP-1/CP2 and TIP, respectively (lanes 2 to 6). Competition with the P5 Inr, P5-60, or LeI Inr, known to bind the YY1 protein (49), did not significantly alter the binding pattern (Fig. 3A, lanes 7 to 9) and neither did the addition of the TEF1 start site region (lane 11); weak competitions of TIP with the TEF1 oligonucleotide and complex 1 with P5-60 are observed (Fig. 3A and D, lanes 8 and 11). On the other hand, the AdML Inr inhibited TIP to some extent (lane 10) and the DraI oligonucleotide inhibited LBP-1/CP2 efficiently (lane 12). Negative results were obtained with oligonucleotides containing a bona fide E2F binding site (a site at −50 of the E2F promoter [22]) and λ5 Inr sequences (35)

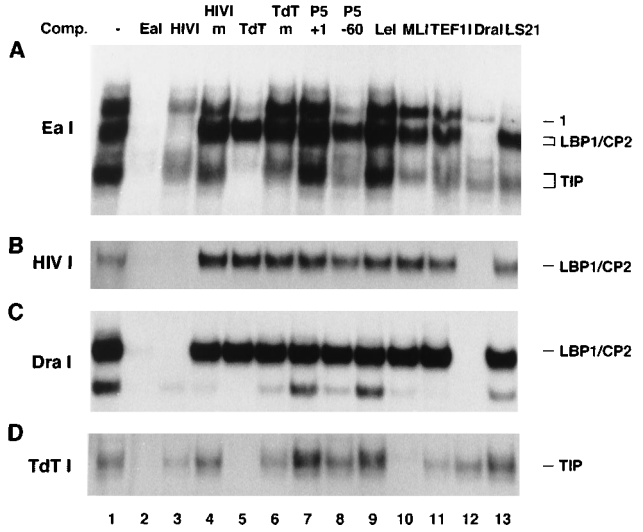


FIG. 3. EMSA of Ea Inr-binding proteins with different Inrs. (A) The four bands obtained with 5 μg of CH27 nuclear extracts and the Ea Inr oligonucleotide (bands 1 to 4) were competed with oligonucleotides (20 ng, 200-fold excess) corresponding to different wild-type and mutant Inrs, as indicated above the lanes. The sequences of each oligonucleotide are indicated in Table 1. The reaction was started by the addition of the nuclear extracts to a mix containing both the cold competitor and the labelled oligonucleotides. (B to D) The scheme of the experiment was as for panel A, except that labelled HIV Inr, DraI, and TdT Inr oligonucleotides were used, respectively, together with 5 μg of CH27 nuclear extracts. m, mutant oligonucleotide.

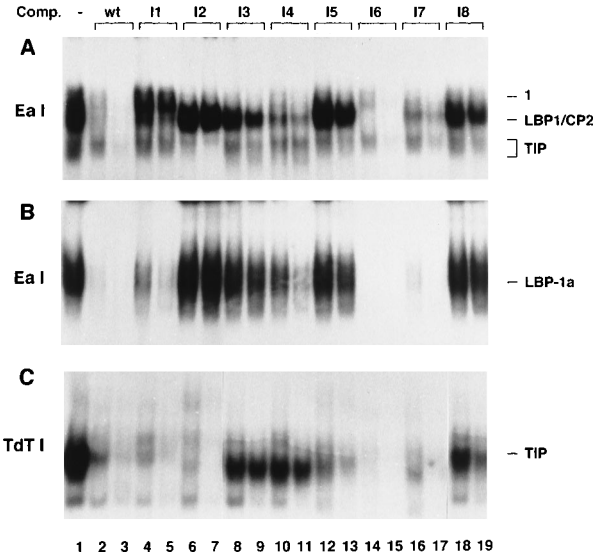


FIG. 4. EMSA of Ea Inr-binding proteins with Ea Inr mutants. (A) The different Inr-binding proteins detected with 5 μg of CH27 nuclear extracts and the Ea Inr were competed with different amounts (50-fold excess, even-numbered lanes; 200-fold excess, odd-numbered lanes) of cold oligonucleotides containing mutations identical to the ones used in the in vitro transcription experiments. The incubation was started by the addition of the nuclear extracts to a mix containing both the cold competitor and the labelled oligonucleotides. (B) Same as for panel A, except that 5 ng of recombinant LBP-1a was used with the Ea Inr oligonucleotide. (C) Same as for panel A, except that the labelled oligonucleotide was the TdT Inr. wt, wild type.

binding MYC/MAX (data not shown). Finally, the EaLS21 mutant oligonucleotide inhibited only band 1.

Subsequently, we did cross-competition analysis with labelled HIV Inr (Fig. 3B), DraI (Fig. 3C), and TdT Inr (Fig. 3D) oligonucleotides. The picture emerging from this panel of EMSAs is in agreement with the results obtained with the Ea Inr oligonucleotide: TIP binding to TdT is inhibited by the Ea Inr and AdML Inr (Fig. 3D, lanes 2 and 10). LBP-1/CP2 binding to HIV Inr or DraI cross-compete with each other and with Ea Inr (Fig. 3B and C, lanes 2, 3, and 12); in addition, the DraI shows a lower band that is also abolished by several competitors, i.e., the Ea Inr, HIV Inr, TdT Inr, AdML Inr, and TEF1 Inr (Fig. 3C, lanes 2, 3, 5, and 10 to 12). As expected, the EaLS21 oligonucleotide was unable to abolish any of these bands. In conclusion, the Ea Inr is able to interact only with a subset of Inr-binding proteins, LBP-1/CP2 and TIP, but not with others, such as YY1, E2F, and MYC/MAX.

EMSA of Ea Inr-binding proteins with Ea Inr mutants. We next tried to correlate the transcriptional activity of the different mutants described before with the binding profiles of TIP and LBP-1/CP2. We did EMSA competition analysis with a set of oligonucleotides carrying the same mutations that were assayed for in vitro transcription (Table 1). Figure 4A shows the results of such experiments, using two different concentrations (50- and 200-fold excesses) of the competing oligonucleotides and two labelled fragments (Ea Inr in Fig. 4A and B; TdT Inr in Fig. 4C). The Ea Inr was incubated with CH27 nuclear extracts—the same as those used for the functional in vitro transcriptions—in the experiment whose results are shown in Fig. 4A, to look for the complete binding pattern, while we used recombinant LBP-1a in the experiment whose results are shown in Fig. 4B, to better visualize this complex; in the latter case, identical results were obtained with LBP-1b and LBP-1c (data not shown).

(i) Band 1. Band 1 is abolished by all oligonucleotides except I1, suggesting that the sequences necessary for binding are on the left side of the Ea Inr.

(ii) LBP-1/CP2. Mutants I2, I3, I5, and I8 are crippled to background levels (Fig. 4A and B, compare lanes 1 with lanes 6 to 9, 18, and 19); I1 and I6 are as good as the wild-type oligonucleotide (actually better in the case of I6). I4 and I7 compete with approximately fivefold-lower affinity but are still considerably better than the noncompeting oligonucleotides (compare lanes 1 to 3 with lanes 10, 11, 16, and 17). These results indicate that the LBP-1 binding site is extended over 12 to 15 bp across the +1 area and that mutant I4 splits the site in two, between I2, I3, I8 (TCTGCT) and I5, I7 (TCTGC). It has been shown that the LBP-1/CP2 HIV site is composed of two repeats [(T/A)CTGG] separated by a 5-bp linker of variable sequence (56): this interpretation is consistent with mutagenesis studies on other sites and with the data presented here (Fig. 2C).

(iii) TIP. As can be seen in Fig. 4A and C, the I3, I4, and I8 oligonucleotides do not bind these complexes at all (lanes 8 to 11, 18, and 19); I2, I6, and I7 behave like the wild type (better in the case of I6), while I1 and I5 have somewhat decreased binding (the former is best seen on the Ea oligonucleotide; compare Fig. 4A, lane 1 and lanes 4 and 5). Therefore, the binding site for these complexes is centered around sequences mutated in I8, I3, and I4, from +1 to +7 (GCCTCAG), and corresponds to a stretch showing homology to the TdT Inr (CCCTCAT) and the AdML Inr (TCCTCAC) (underlined bases differ from the corresponding bases in the Ea Inr). This result suggests that these nucleotides are responsible for the cross-competition observed between the TdT Inr and the Ea Inr oligonucleotides and, partially, with the AdML Inr oligonucleotide.

Altogether, the binding data show several discrepancies with

respect to the functional in vitro experiments, since the transcription profile does not match the binding of either LBP-1 (good binding of crippled I4 and I6 and weak binding of higher I2) or TIP (I5 and I6 bind well and I5 binds much better than I3, but the latter is stronger in transcription). We can conclude that the Ea Inr activity can be ascribed to none of the Inr-binding proteins described so far.

TFIID binding correlates with Ea Inr activity. It has recently been shown by extensive mutagenesis and binding studies on the TdT Inr that the binding of recombinant YY1 does not determine Inr activity, which is, rather, dependent on specific interactions made by TFIID in the start site region (26, 28). Because a positive role for Inr-binding promoters could not be established for Ea, and since Ea and TdT Inrs appear to be functionally related, this finding prompted us to investigate whether TFIID is able to bind to the weak Ea TATA box, whether the Inr element is important for TFIID interactions, and whether binding of TFIID to Ea promoter sequences could explain the in vitro transcription data. For this purpose, we used an EMSA system in agarose gels that allows visualization of the large multisubunit TFIID complex (66). TFIID fractions were immunopurified from HeLa cells with an anti-TBP monoclonal antibody, according to a protocol which already proved useful in the identification of factors able to restore Ea heat-sensitive transcription (35), in the classification of different classes of coactivators, and in the cloning of human $TAF_{II}30$, $TAF_{II}18$, $TAF_{II}20$, and $TAF_{II}28$ (6, 24, 39).

We first wanted to verify the DNA binding capacity of TFIID by using a PCR-generated fragment containing sequences from −74 to +16 of the Ea promoter, which include the TATA box and Inr. Figure 5A indicates that a specific complex is visible in EMSA agarose gels and is inhibited by an excess of cold AdML TATA box oligonucleotide, an Ea TATA box oligonucleotide, and the Ea Inr oligonucleotide containing only the Ea Inr region (Table 1) but not by an Sp1 binding site (Fig. 5A, lanes 1 to 5, respectively); the results obtained with the TATA and Ea Inr oligonucleotides are a clear indication that within this long Ea fragment, TFIID appears to be at least partially associating with both TATA and Inr sequences. The binding affinity of TFIID for the Ea promoter is fivefold lower than that for the strong AdML TATA box, according to binding and competition data (data not shown).

We verified whether such binding was dependent on the Ea TATA box by generating an identical long Ea fragment containing a mutation in the TATA box, previously shown to bind TBP very poorly (35). The wild-type fragment yielded a much stronger band (Fig. 5B, compare lanes 2 and 5 for complex D); this defect was partially compensated for by the addition of highly purified TFIIA (Fig. 5B, compare lanes 3 and 6 for the DA complex), itself a non-DNA-binding protein (Fig. 5B, lanes 1 and 4). Furthermore, and contrary to purified TFIID, recombinant TBP alone was not able to stably bind to the Ea TATA box (Fig. 5C, compare lane 1 with lane 3), while the addition of TFIIA induces the appearance of the TA complex (Fig. 5C, lane 2) as well as modification of the mobility of the D complex (Fig. 5C, lane 4; also Fig. 5B). This result suggests that a polypeptide(s) other than TBP present in TFIID is required to stabilize binding on the Ea TATA-Inr sequences in the absence of TFIIA, since TBP alone is unable to generate a band by interacting with the Ea TATA box. In these experiments, however, we notice that the TA and the DA complexes have similar electrophoretic behaviors in our agarose gel system, an observation that was previously described in other studies (31, 66): this might be due to the remarkable bend introduced in the DNA upon TBP binding (29) or to the fact that TAFs are dissociating from TBP during electrophoresis.

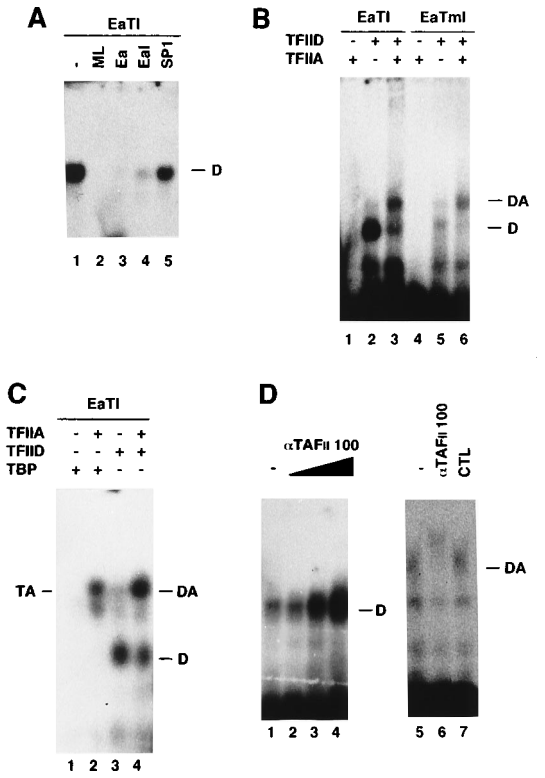


FIG. 5. TFIID binds to Ea TATA-box- and Inr-containing fragments. (A) TFIID binding to the Ea fragment (from −74 to +16) and competition with 100-fold excesses of AdML TATA box cold oligonucleotide (lane 2), Ea TATA box oligonucleotide (lane 3), Ea Inr oligonucleotide (lane 4), and Sp1 oligonucleotide (lane 5). TFIID was added last to a mix containing labelled Ea fragment and cold oligonucleotides. (B) EMSA using TFIIA alone (lanes 1 and 4), TFIID alone (lanes 2 and 5), or TFIID plus TFIIA (lanes 3 and 6) with Ea fragments containing the wild-type TATA box and Inr (lanes 1 to 3) or the mutant TATA box and Inr (lanes 4 to 6); the D and DA complexes are indicated. (C) Recombinant TBP (10 ng) and purified TFIID were incubated alone (lanes 1 and 3) or together with TFIIA (lanes 2 and 4), generating complexes D, TA, and DA. (D) TFIID was incubated alone (lanes 1) or preincubated for 2 h on ice with increasing amounts of purified anti-$TAF_{II}100$ (20, 100, and 500 ng, respectively, in lanes 2 to 4); TFIID and TFIIA were incubated alone (lane 5), with 500 ng of anti-$TAF_{II}100$ (lane 6), or with control anti-NF-YA (lane 7) antibodies, before the addition of labelled DNA.

To ultimately prove that the D complex in our EMSA does contain TAFs, we used an anti-$hTAF_{II}100$ antibody (24) in supershift experiments. Figure 5D shows that preincubation with increasing amounts of purified antibody modified the migration of complex D (lanes 2 to 4) and that of complex DA (lane 6), while no effect was seen with a control, anti-NF-YA, antibody. From this set of experiments we conclude that the D complex in our agarose EMSA is specific, is TATA and Inr dependent, and contains TBP plus additional polypeptides—TAFs—that are associated with TBP. Moreover, in the absence of TFIIA, at least one of these TAFs increases TBP affinity for Ea promoter sequences.

We thus felt confident that such an assay could be used with fragments containing the wild-type TATA box and mutations corresponding to the Inr mutants depicted in Fig. 1 and used for Fig. 4. Figure 6A shows the result of such experiments with the most relevant and discriminatory of the mutants, including in the binding reaction mixture either only TFIID (lanes 1 to 7) or both TFIID and TFIIA (lanes 8 to 14). Strong down mutations are I4, I5, I6, and I8, both for the D complex (Fig. 6A, compare lane 1 with lanes 3 to 6) and, to a lesser extent with

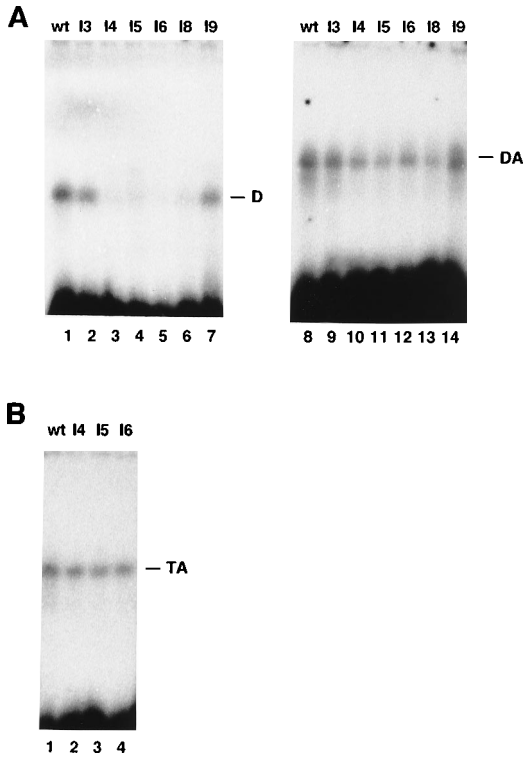


FIG. 6. EMSA of TFIID with Ea Inr mutants. (A) EMSA with TFIID (lanes 1 to 7) and TFIID plus TFIIA (lanes 8 to 14) using Ea fragments containing wild-type (wt) Inr or Ea Inr mutants (as indicated above each lane). (B) Ea wild-type (wt) Inr (lane 1) and mutants I4, I5, and I6 (lanes 2 to 4) were incubated with recombinant TBP (10 ng) and purified TFIIA, generating the TA complex.

the addition of TFIIA, for the DA complex (compare lane 8 with lanes 10 to 13). A small reduction in binding is also found with I3 (Fig. 6A, lanes 2 and 9), while I9 retains full binding capacity (lanes 7 and 14). The differences are particularly evident with the D complex (Table 2), probably because the stabilizing effect of TFIIA on the Ea TATA box renders the DA complex slightly less dependent on Inr sequences. As a further control, we checked the affinity of the three most-crippled mutants, I4, I5, and I6, for the TA complex, formed by the association of TFIIA and recombinant TBP, known to recognize only TATA boxes. Figure 6B shows that no significant difference is observed with Inr mutants compared with the wild-type Ea fragment.

We can conclude that binding of TFIID to the Ea promoter is dependent both on the TATA box and on the Inr and that within the latter element, there is a good correlation between TFIID binding and transcriptional activity, with the exception of I8, a G-to-T transversion at +1, at the edge of the TFIID Inr-binding region. I8 shows reduced TFIID binding but is normal in transcription assays.

Behavior of Y-box point mutants. To help explain this apparent discrepancy, we introduced in the PE3 vector used for in vitro transcriptions single-base-pair mutations within the conserved Ea Y box, known to variously affect the binding of NF-Y (12). Complete mutation of this 10-bp element virtually abolished Ea transcription in different assay systems (3, 17), as did challenging in vitro transcriptions with anti-NF-Y antibodies (34). These observations are true for all MHC class II promoters (3, 17). In vitro transcription analysis of mutants harboring transversions in the conserved Y-box sequence, PEm16 and PEm17, shows essentially no difference with respect to the wild-type vector (Fig. 7A, compare lanes 1, 4, and 5). PEm9 and PEm12, in which mutations affect the central CCAAT sequence, have two- to threefold-reduced transcriptional activity (Fig. 7A, lanes 2 and 3). Figure 7B highlights the differences in NF-Y binding efficiency, as tested in EMSA, and Ea transcriptional activity of single-base-pair mutations, strongly indicating that in the context of this promoter, in vitro DNA binding is much more sensitive to subtle changes in DNA sequence, while more radical mutations are needed in functional assays. Thus, we conclude that in terms of susceptibility to single-base-pair mutations, TFIID behaves very much like NF-Y on the Ea promoter.

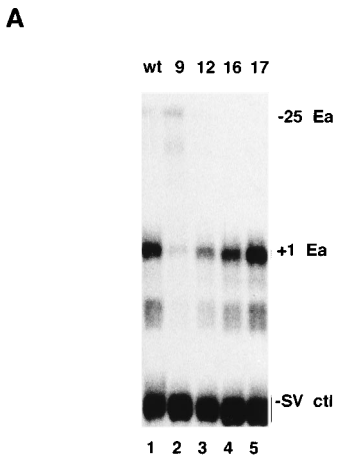
DISCUSSION

In this study, we have performed a functional dissection of the start site region of the Ea promoter and found that this important element contains a TdT-like Inr that appears to be unique among Inrs, in that it does not overlap with the Cap site but is, rather, placed in a transcribed region; this element is critically dependent on interactions with the TFIID complex, despite high-affinity binding of Inr-binding proteins.

TABLE 2. Ea Inr: summary of DNA-binding and transcriptional data

Inr	Sequence[a]	Binding affinity[b]				Transcription efficiency
		TIP	LBP-1	D	DA	
TdT	−11 GCTCGGCCCTCATTCTGGAG +9					
WT[c] Ea	−6 AATTCTGCCTCAGTCTGCGA +14	+++	+++	100	100	100
I1	CCGTCTGCCTCAGTCTGCGA	++	++	92 ± 9	105 ± 10	80 ± 8
I2	AATGACGCCTCAGTCTGCGA	+++	−	78 ± 12	89 ± 12	35 ± 5
I3	AATTCTGTGCCAGTCTGCGA	−	−	48 ± 6	66 ± 5	33 ± 6
I4	AATTCTGCCTGTCTCTGCGA	−	++	13 ± 4	40 ± 3	9 ± 2
I5	AATTCTGCCTCAGAGGGCG	++	−	17 ± 5	36 ± 4	15 ± 2
I6	AATTCTGCCTCAGTCTCGA	++++	++++	16 ± 3	46 ± 5	13 ± 3
I7	AATTCTGCCTCAGTCTAAAA	+++	−	ND	ND	45 ± 7[d]
I8	AATTCTTCCTCAGTCTGCGA	−	+	28 ± 10	53 ± 9	90 ± 15
I9	ACCCTCATTCTAGTCTGCGA	+++	−	92 ± 8	104 ± 9	510 ± 45
LS21	ACGGATCGATGAGTCTGCGA	−	−	15 ± 4	29 ± 5	5 ± 2

[a] Underlined nucleotides represent mutations. Boldface nucleotides represent major start sites. Doubly underlined nucleotides are identical in the TdT and Ea Inrs.
[b] ++++, >100%; +++, 50 to 100%; ++, 25 to 50%; +, 10 to 25%; −, <10%.
[c] WT, wild type.
[d] This value refers to the Ea +1 signal; when both the +1 and +7 signals are taken into account, the overall value for I7 is 145 ± 27.



B

		NFY binding affinity	Transcription
wt	CTGATTGGTTAA	100	100
PEm9	CTGCTTGGTTAA	< 1	35 +/- 5
PEm12	CTGATTTGTTAA	< 1	45 +/- 10
PEm16	CTGATTGGTTCA	20	95 +/- 6
PEm17	CTGATTGGTTAC	25	110 +/- 13

FIG. 7. Effect of Y-box point mutations on Ea in vitro transcription. (A) In vitro transcription experiments employing the PE3 vector, containing the wild-type (wt) Ea promoter (lane 1) and vectors, PEm9, PEm12, PEm16, and PEm17 containing single-nucleotide changes in the Y-box sequence (lanes 2 to 5). The normal Ea +1 signal, as well as the spurious −25 and SV40 internal control signals, is indicated. (B) The Y-box sequences of the wild type (wt) and mutants used in panel A are shown, together with transcription efficiencies and relative affinities for NF-Y (12). The reverse CCAAT sequence is boxed. Mutations are underlined.

Inr-binding proteins. In a previous study, we detected four major activities binding to the Ea Inr; here we characterized their binding requirements and identify one of them. Band 1 is of little interest, since it binds to a functionally neutral region 5′ of Ea position −6 (56).

Band 2 is the ubiquitous factor LBP-1, whose genes have been cloned in mice and humans (32, 62) and which has been shown to have homology in the DNA-binding and dimerization domains with *Drosophila* Elf-1/NTF1/GRH (4, 14). Multiple forms of this protein exist, arising from alternative splicing (62). By EMSA we identified the binding site as two direct 5-bp repeats (TCTGC) spaced by 5 nucleotides; a mutation in the 5-bp linker (I4) is well tolerated. These data are in good agreement with previous mutagenesis studies performed on the HIV, α-globin, and γ-fibrinogen sites (32, 62). We also identified a new protein, TIP, binding to the TdT Inr: our EMSA competition experiments indicate that TIP, band 3-4, is neither YY1, because of the lack of binding with high-affinity sites P5-1, P5-60, and LeI (49), nor is it E2F or MYC/MAX, whose binding sites also do not compete. It might, however, be related to TFII-I or USF, proteins that bind to TdT and AdML Inrs, but the lack of competition with HIV Inr, which also binds USF (13), is at odds with this conclusion. The nucleotides recognized by TIP are a pyrimidine stretch (GCCTCA) corresponding to the 5′ part of the TdT homology (CCCTCA) and to sequences in the AdML Inr (TCCTCA). The in vitro transcription data indicate that although the LBP-1/CP2 and TIP binding sites overlap critical Inr sequences, they cannot fully account for the activity of this element. Moreover, the addition of either recombinant LBP-1a, LBP-1b, or LBP-1c to our in vitro system led to no change in activity, as did preincubation of purified anti-LBP-1 antibodies with the CH27 transcription extracts (33a). The results presented here concerning the natural Ea promoter argue against the hypothesis that Inr-binding proteins have a specific role in Inr activation and are reminiscent of the conclusions reached by extensive mutagenesis studies performed with the TdT Inr, using hybrid constructs containing upstream Sp1 sites. It was clearly established that no relationship between YY1 and Inr activity exists, despite the fact that the nucleotides necessary for YY1 interaction overlap with the core TdT Inr (26); in the case of the Ea promoter, we extended such observations to the previously undetected TIP protein, whose binding site is shared between TdT and Ea, and to the HIV Inr-binding protein LBP-1/CP2.

Do such proteins play a role in this system? Conservation of the LBP-1/CP2 binding site around the initiation regions between mouse Ea and human Dra points to a possible role that might be underscored by the reductionistic approach taken here. Recent experiments performed on globin promoters, both avian and human, indicate that LBP-1/CP2 is part of a heteromeric complex, together with a tissue-specific erythroid protein, which is involved in the developmental stage-specific regulation of γ-β promoters (in humans) and β^A (in chickens) (references 2 and 25; see reference 18 for a review). In the latter case, it has been proposed that it might help the binding of the TFIID complex (2). Given the common general features of MHC class II and globin genes (both are organized in gene clusters; both have locus control regions (LCRs), tissue-specific promoters, and enhancers; and both are expressed in a stage-specific way), one could speculate that LBP-1/CP2 is not important in the determination of the overall promoter strength but rather in the communications between the LCR and the promoter or in facilitating the access to other promoter-specific activators in a stage-dependent way.

TFIID and Ea Inr activity. Functional experiments presented in this study clearly indicate that the Inr is an important element in the Ea promoter and that mutations are almost as lethal as elimination of the conserved X and Y boxes. Using immunopurified TFIID and several Inr mutants, we have shown that a good correlation exists between TFIID binding and Inr activity. The only exception is mutant I8, bearing a single-base-pair mutation at +1, that has normal transcriptional activity with reduced TFIID binding. However, we have shown that point mutations in the binding site of NF-Y, an essential MHC class II activator that is critically susceptible to any single-base-pair mutation in EMSAs (12), only show a very modest transcriptional effect in the context of the whole promoter. This can best be explained by assuming that protein-protein interactions between essential activators can, to some extent, compensate for the reduced affinity of any given factor for DNA. In keeping with this interpretation, NF-Y has been shown to help the interactions of other proteins, RF-X in the Dra promoter (47) and C-EBP in the albumin promoter (40), with nearby sequences.

The TFIID-interacting stretch shows extended homology with the TdT Inr (Table 2): placing the 10-bp core TdT Inr in the −6 to +4 Ea position considerably increases promoter strength, probably because in such a construct there is a duplicated Inr, without altering start site selection. This result indicates that upstream activators, mainly RF-X/NF-X and NF-Y, which are strictly necessary for Ea transcription, can work perfectly with the TdT-like Ea Inr configuration: the mechanisms of Inr activation are therefore probably similar. This combination is different from that observed in other Inr-containing promoters, where Sp1 binding sites seem to predominate (63). The two promoters share other resemblances, since both are tissue specific, their expression patterns partially

overlap (3, 16, 17), and they lack a functional TATA box (41, 50, 51, 56, 64). However, notable differences do exist. (i) While TdT seems to completely lack a TATA box and TBP binding to the natural −30 region is negligible (65), the Ea TATA-like sequence interacts normally with TBP, TBP/TFIIA, TBP/TFIIB (35), and TFIID; consequently, while TFIID must rely exclusively on Inr sequences in TdT, both the Ea TATA-like and Inr sequences are involved in TFIID binding, but only interactions with the latter are crucial. (ii) While the TdT Inr possesses a low but detectable intrinsic activating capacity (51), the Ea Inr is absolutely dependent on upstream activators (3, 17, 34, 56). (iii) Unlike TdT, whose Inr overlaps the +1 signal, the key Ea Inr sequence is displaced 6 nucleotides on the 3′ side of the major start site.

Several studies described extended footprints of TFIID over the transcriptional initiation regions, in addition to TATA box protections (15, 20, 21, 45), as opposed to the shorter TATA-centered footprints obtained with TBP (19, 59–61); these observations led to the hypothesis that additional TBP-associated proteins, TAFs, were responsible for Inr interactions. Evidence for this has recently been obtained. (i) At least one of the *Drosophila* TAFs, $TAF_{II}150$, is able to directly interact with the AdML promoter in the +10 region, without the need of TBP/TATA (54, 55). (ii) Binding site selection studies with *Drosophila* TFIID identified a 6-bp consensus [(g/a/t)(c/t)A(t/g)TG] centered around the initiation start site (45), which is responsible for TFIID binding in addition to the TATA box. (iii) The *Drosophila* HSP70 +10 region has been shown to be bound by TFIID and to interact specifically with a 150-kDa polypeptide in purified dTFIID (52) and with $dTAF_{II}150$ and $dTAF_{II}250$ (54). For all these reasons, it seems reasonable to hypothesize that the equivalent mammalian factor might be responsible for the key DNA contacts made by our immunopurified TFIID at the Ea Inr.

In summary, for Ea we provide evidence against a specific role of Inr-binding proteins in transcription and in favor of a TdT-like scenario, in which TFIID would make key DNA interactions at the Inr, possibly through the equivalent of $dTAF_{II}150$, and would be held in place by essential protein-protein contacts with upstream factors.

Inr in other MHC class II promoters? Although some of the promoters of MHC class II genes have TATA-like sequences, the lack of conservation of the TATA element in these promoters, which otherwise share the invariant Y and X and the quasi-invariant X2 elements, is rather puzzling (3, 17). The Ea TATA-like box binds TBP and TFIID normally, but linker-scanning mutants of both Aa and Ea promoters prove that this element is functionally expendable, both in vitro and in vivo (10, 35, 56), thus classifying them among TATA-less promoters.

The molecular dissection of the Ea Inr and identification of a TFIID binding site prompted us to search for such a sequence in the promoters of other MHC class II genes. Alignments of several such promoters in the corresponding region indicate that a best fit is obtained within a TdT-like Inr consensus, (t/c)(a/g)CA(g/t)(t/a)(c/t)(t/c) (Table 3), which is also potentially a good TFIID binding site (45). For most genes this stretch overlaps the +1 signal, but for others, such as Aa and Dqa, the situation is similar to that for Ea, since the supposed Inr is placed within transcribed areas. Differences in this stretch among MHC class II promoters are certainly greater than those for their X and Y boxes, but this is likely to reflect the far looser sequence requirements observed for TFIID at Inrs (45), compared with the strict sequence specificity of NF-Y and RF-X/NF-X (12, 30, 46). Because of the similar architecture of all MHC class II promoters and the presence in all of them of Ea- or TdT-like Inr sequences, we speculate that all MHC class II genes use this element for promoter function, possibly with a common set of TAFs. TBP binding to the TATA-like sequence would be possible and would help stabilize the complex, but it is not crucial, as it is for "true" TATA-containing promoters. This hypothesis does not exclude the possibility that in some promoters, like Dra, the TATA box might help stabilize TFIID and play a significant role in determining promoter strength and start site selection. Interestingly, the TdT Inr mutational analysis has been performed on a synthetic promoter containing Sp1 as an upstream activator, and it has been shown that Sp1 helps TFIID binding (28). Since both Sp1 and NF-Y have Q-rich activation domains, one possible model that is currently under investigation envisages close interactions between the CCAAT box-binding protein NF-Y and TFIID: indeed, observations from our lab indicate that NF-Y is able to specifically interact with TFIID, and unpublished results cited in reference 9 point to interactions between CBF-B/NF-YA and *Drosophila* $TAF_{II}110$. The availability of the different NF-Y subunits and TAFs together with the EMSA system described here will certainly be useful in understanding the interplay of protein-protein interactions between these multisubunit complexes at the molecular level.

TABLE 3. Sequences of MHC class II promoters in the initiation region

Gene	−25 to −30 sequence	Inr sequence
Ea	ATTTTAAT	−6 AATTCT**G**CCTCAGTCTGCGA +14
Aa	TTTGGTTT	−2 TG**G**ATCCTCACAATCTCTTA +18
Dra	ATTTTAAT	−6 TCTTTTCTTTT**A**TTCTTGTC +14
Dqa	CAGATTTC	−4 AATT**G**CTCTACAGCTCAGA +15
Dpa	CCCATTTC	−12 CCCACAGTTTCA**G**TCTCATC +8
Eb	TTATTATC	−9 GATTAAGTT**A**CAGTCTGAAG +11
Ab	TGGGATTT	−11 ATACTTCACGC**A**CTTTTCTC +9
Drb	TATAATTT	GGAGAGGGGTCATAGTTCTC
Dqb	TTTTTATT	−9 CCAGGT**A**CA**T**CA**G**ATCCATC +11
Dpb	CTTGGGTT	−14 ATATTTCAAACAGG**A**GCTCC
MHC class II Inr consensus		TTTC**A**GTCT CCA TATC
dTFIID consensus (45)		GCAGTG AT T T
Inr consensus (7, 26)		CCTC**A**NTCTG C AT

ACKNOWLEDGMENTS

We thank S. Ottolenghi and A. Barberis for critically reviewing the manuscript.

This work was supported by a Pharmacia/FCE grant to R.M., Progetto Finalizzato ACRO and AIRC grants to S. Ottolenghi, and CNRS and INSERM grants to L.T. and in part by Public Health Service grants CA42567 and AI27327 to R.G.R. and by grants from the Pew Charitable Trusts to Rockefeller University. M.B. is supported by a Milano Ricerche fellowship; J.-B.Y. is supported by an AmFAR research grant.

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