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A Novel Transcriptional Initiator Activity of the GABP Factor Binding ets Sequence Repeat From the Murine Cytochrome c Oxidase Vb Gene

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The murine cytochrome c oxidase (COX) subunit Vb mRNA contains heterogeneous 5′ ends mapping to +1, +6, +12, +17-22, +24-29, and +32-35 positions of the gene. We have previously shown that initiation of RNA at the +1 position of the promoter depends upon a YY-1 (NF-E1) binding initiator motif. In this article we show that the GABP factor binding duplicated ets motif, GTTCCCGGAAG, at +16 to +26 position functions as an independent initiator for transcription of RNAs mapping to the +18-19 and +23-26 regions. The initiation region ets sequence repeat (ets-ets sequence) can drive the transcription of the CAT reporter gene. The upstream ets site of the ets-ets sequence exhibits a low affinity for binding to purified GABP factors, whereas the downstream site exhibits high affinity. S1 analyses of RNA from transfected COS cells demonstrate that the initiation region ets-ets sequence can accurately initiate transcription at the +18-19 and +24-25 regions. Transcriptional initiation at these two positions, but not at +1, +12, and +31-32 positions, show a selective dependence for intact downstream ets site and GABP α and β factors as tested in an in vitro reconstituted system. The activities of both COX IV and COX Vb single site ets initiators are induced in vivo by coexpression with GABP α and β cDNAs. These results provide evidence that the 5′ heterogeneity of the COX Vb mRNA is largely due to independent transcription initiations at multiple initiator motifs that bind to various transcription factors.

Transcription initiator ets motif Cytochrome oxidase Vb gene GABP factor

TRANSCRIPTIONAL start sites of a number of genes have been described that fall within pyrimidine-rich PyCAPy sequence motifs, termed Inr (33,44). It is becoming increasingly apparent that these Inr motifs together with the upstream TATA or other activator elements, like GC boxes, are involved in recruiting the basal transcription factors to the initiation complex (6,22,33). Studies with synthetic promoter constructs containing the TdT Inr with an upstream TATAA motif have shown that mutations in the Inr cause reduced promoter strength, as well as altered transcription start sites (22,33). Recent studies also suggest a functional interaction of the Inr motif with a component of the TFIID complex (18,26,27, 37). In one case, a complex containing the *Drosophila* dTBP, *Drosophila* $TAF_{II}150$, and human TAFII250 yielded an extended region of DNAse I

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footprint that overlapped the TATA box, the Inr, and downstream sequence up to +38 position of the AdML promoter (37). In TATA-less promoters, it has been postulated that the Inr or analogous motifs from initiation region (IR[3]) may have important roles in recruiting TBP (45), although the roles of the various IR binding proteins in the recruitment of basal transcriptional factors have yet to be established. Recently Roy et al. (15,29) showed that TFII-I facilitates the recruitment of TBP to the AdML promoter, a requirement for the formation of a preinitiation complex in an alternate pathway independent of factor TFIIA.

In a number of promoters, the putative Inr sequence motifs around the start sites that exhibit various degrees of sequence divergence from the TdT Inr (6,22), appear to be sufficient for basal level of transcription and also for positioning of transcription initiation sites. Promoters lacking a consensus Inr-type sequence, including a 22-bp tandemly repeated sequence containing the GABP factor binding ets sequence motif (GGAA) from the transcription start sites of the cytochorme c oxidase (COX) IV gene (7), PEA1 and PEA3 binding sequence motifs from the polyomavirus late promoter (43), and an Sp1 factor binding motif from the human Ha-ras gene (20), have been reported to function as autonomous initiator elements. To distinguish between consensus Inrs and functional IR elements lacking the PyCAPy motifs, we will refer to the latter more generally as "initiator elements."

Studies on the interaction of specific transcription factors with the pyrimidine-rich motifs, exhibiting initiator activity, have demonstrated a functional role for transcription factor YY-1 (also referred to as NF-E1) in the transcription initiation from adeno-associated virus type 2 P5+1, mouse COX Vb, and the human leukocyte interferon gene initiator elements (2,31). Similarly, binding of transcription factor TFII-I to the AdML gene (15,29), HIP-1/E2F to the dihydrofolate reductase gene (4,) and USF to the HIV-1 gene (15) initiator elements have been shown to be important in transcription activation or start site positioning.

In contrast to the view that structurally different transcription factor binding motifs from IR may function as transcription initiators, some studies suggest that the Inr may be a functionally distinct entity and binding of gene-specific transcriptional factors to the IR sequence motifs may be inconsequential for their transcription initiator function. In support of this view, Javahery et al. (16) have recently analyzed a series of mutations targeted to the Inr motifs from both adeno-associated virus P5+1 and DHFR promoters, cloned downnstream of a cryptic TATA motif at positions analogous to the adeno P5+1 promoter. The results show that various mutated Inr sequences exhibit widely variable YY-1 and E2F factor binding affinities, which did not correlate with their Inr activities. Thus, currently two divergent models for the initiator function are emerging. According to one model, the Inr is unique both structurally and functionally, and it interacts with a universal protein as part of the pathway for recruiting the basal transcription factors. Binding of YY-1 (NF-E1), E2F, and other factors simply activate or augment the activity of the Inr (16). In the second model, initiator elements with heterogeneous sequence properties binding to various gene-specific transcription factors independently, or in collaboration with the upstream activator motifs, are involved in recruiting TBP and other basal transcription factors.

The COX Vb gene encodes RNA with heterogeneous 5′ ends (1) that map to distinctly different protein binding motifs and thus provides a unique system for investigating the mechanisms of transcription initiation from a cluster of closely organized transcription initiator sequence in their natural setting. In this study, we show that the GABP binding ets sequence repeat at the +16 to +26 position exhibits an initiator-like activity under both in vivo and in vitro conditions. These results confirm and extend our recent results on the possible initiator function of the COX IV IR repeat (7), in addition to suggesting that multiple transcription initiations on the COX Vb gene are directed by multiple and structurally distinct initiator elements.

MATERIALS AND METHODS

Cell Culture and Transfection

Conditions for the growth of 3T3 mouse fibroblast and COS monkey kidney cells have been described previously (1,8). Cells were transfected with 5 μg of CsCl purified test plasmid and 1 μg of β-galactosidase expression plasmid by the calcium phosphate precipitation method (13). Overexpression of GABP α and β was performed by cotransfection of 1 μg of each CMV-GABP expression plasmid. The total amount of CMV vector was normalized to 2 μg by addition of appropriate amounts of CMV vector lacking insert. Cells were harvested at 48 h after transfection; the CAT activities of cell extracts were assayed using

[^{14}C]choloramphenicol and normalized to the β-galactosidase activities (14) as described previously (2,8).

Plasmid Construction

All constructions were done in an enhancerless and promoterless CAT basic plasmid (pCAT basic, Promega Biotech Corporation). Generation of the −17CAT (sequence −17 to +40) has been described previously (2). The −6CAT DNA was prepared by cloning the PCR-amplified DNA sequence −6 to +35 in pCAT basic plasmid. The details of COX IV IR repeat sequence motif WT, with both intact ets sequence and Mut1, which has mutated upstream ets site cloned in pCAT basic plasmid, were described recently (7). The following synthetic oligonucleotides corresponding to COX Vb basal promoter elements used in this study were constructed with Sal I and Xba I specific overhangs, and cloned in the pCAT basic plasmid:

ets-ets: 5′-tcgacGCCTGTTCCCGGAAGTG
CATt-3′
ets-mut: 5′-tcgacGCCTGTTCCCG**ACT**GTG
CATt-3′
mut-ets: 5′-tcgacGCCTGATCGCGGAAGTG
CATt-3′
mut-mut: 5′-tcgacGCCTGATCGCG**ACT**GTG
CATt-3′
Minimal ets-ets: 5′-tcgacTGTTCCCGGAAGT-3′
Sp1/ets-ets: 5′-tcgacCGTCCCGCCCTGCTTGC
TCAGCCTGTTCCCGGAAGTGCATt-3′
2xSp1/ets-ets: 5′-GGGCGGGCTGATCAGCATG
CATGCCTGCAGGTCGACAGCTTGC
ATGCCTGCAGTCGACGCCTGTTCCC
GGAAGTGCATT-3′

Gel Mobility Shift Assays for DNA–Protein Binding

Nuclear extracts from COS and 3T3 cells were prepared essentially as described (32). Protein–DNA complexes were detected with double-stranded DNA probes labeled either by the Klenow extension of a specific 3′ end using an appropriate [α-^{32}P]dNTP or 5′ end labeled by T4 kinase reaction using [γ-^{32}P]ATP. Approximately 0.1–0.2 ng of the probe (10–20,000 cpm) was incubated with bacterially expressed, purified GABP α (100 ng) and β (500 ng) or 5–8 μg of nuclear extract proteins from indicated cells, essentially as described (32). Poly(dI-dC) was included in the binding reactions at varying concentrations (2 μg for purified proteins and 4 μg for the nuclear extracts). The binding reactions were carried out for 25 min at room temperature and the products were resolved in a 4% acrylamide gel in low salt (0.25X TBE) buffer.

In Vitro Transcription Assays

In vitro transcription was performed using HeLa cell nuclear extract prepared according to Dignam et al. (11). Reactions in 20 μl final volumes were carried out in 6 mM Tris-acetate, 4 mM HEPES (pH 8.0), 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 20% glycerol, 60 mM potassium glutamate, 2 mM spermidine, 10 μg poly(dG:dC), 2% polyvinylalcohol, 100 μg of HeLa cell nuclear extract, and 400–450 ng of template DNA. The contents were preincubated on ice for 15 min, supplemented with 500 nM each of rNTPs, and the reaction was carried out for 30 min at 30°C. Reactions were terminated by adding 80 μl of stop buffer (3.125 M NH_4CH_3COO, 100 μg/ml yeast tRNA) and RNA was recovered by phenol extraction followed by ethanol precipitation. The RNA was annealed with 1 ng of ^{32}P end-labeled CAT-rev primer (5′-CGGTGGTATATCCAG-3′) and assayed by primer extension with 10 units of AMV reverse transcriptase as described previously (2).

Nuclear extracts were depleted of GABP α and β factors either by immunodepletion or DNA affinity chromatography. For immunodepletion, 50 μl of nuclear extract was incubated on ice for 2 h with 25 μg of specific antibody or preimmune serum protein, and the antigen–antibody complexes were cleared by adsorption to protein A Sepharose (10). The α and β subunit depletions were complete as tested by the failure of ets-ets IR repeat DNA to form a complex in gel mobility shift assays (results not presented). In the second method, multimerized ets-ets DNA or ets-mut DNA were covalently conjugated to CNBr-activated Sepharose (17) and used for factor depletion essentially as described by Kawaguchi et al. (17,41), excepting that batch purification using 200 μl of the HeLa nuclear extract and 200 μl of DNA-bound resin was employed. A nearly complete depletion of the GABP α and β required three successive passages through the affinity matrix. The GABP α and β factors from HeLa nuclear extract were purified by affinity binding to DNA–Sepharose essentially as described for the purification of the human factors (41).

S1 Nuclease Analysis

S1 nuclease analyses of RNA from mouse liver, heart, kidney, and brain were carried out essen-

tially as described by Berk and Sharp (3). A 187 nucleotide long S1 probe (+63 to −124) was generated using 5′ end-labeled synthetic primer 5′ - CAGGGCCTGCGCCGCCAAAGCGC - 3′, which is complementary to sequence +41 to +63 of the COX Vb gene, as described previously (1). S1 protection of RNA from cells transfected with various CAT constructs was carried out using probes generated by the Klenow extension of 5′ end-labeled CAT-Rev primer 5′-CGGTGGTA TATCCAG-3′, complementary to CAT gene sequence 2331–2345. Details of probe preparation, annealing with RNA, digestion with S1, and analysis of protected fragments have been described previously (1).

RESULTS

Heterogeneous COX Vb mRNA 5′ Ends Mapping to Different Protein Binding Motifs

Previous studies from our laboratory demonstrated a 5′ heterogeneity of the mouse liver COX Vb mRNA (1). In the present study, the precise 5′ ends of mRNA from different tissues, including liver, brain, kidney, and heart, were compared by S1 nuclease mapping. As shown in Fig. 1, RNA from different tissues show nearly identical 5′ termini, although the relative intensities of bands with RNA from different tissues markedly vary, reflecting mRNA abundance. The heterogeneous transcripts in each case map to +1, +6, +12 sites in addition to three major clusters at positions +17–22, +24–29, and +32–35 of the gene. These map positions are similar to the 5′ termini detected by the primer extension analysis presented in an earlier article (1). The nucleotide sequence (−17 to +40) from IR of the COX Vb gene is presented in Fig. 2A, and the major species of mRNA 5′ ends determined by the S1 nuclease analysis indicated by the arrows. The −17 to +40 region of the COX Vb promoter contains a YY-1 (NF-E1) site fused to an Sp1 site around the +1 position (2). Flanking both sides of the Sp1/YY-1 motif there are GGAA sequences, which is the core binding motif of the ets family of transcription factors. A single ets motif is found at −13 to −8 position. At +16 to +26 position, two motifs are tandemly repeated in a head-to-head orientation, designated as ets-ets site. Based on the sequence requirements for GABP factor binding reported by Brown and McKnight (5), the motif at the −13 to −8 and also the downstream site (+21 to +26) of the duplicated ets-ets motif show consensus predicted for high-affinity GABP factor binding sites. The upstream ets site of the ets-ets motif located on the antisense strand shows a weak consensus for GABP binding. Three species of downstream transcripts (+6, +12, and the cluster at +32–35) map to sequences that bind to as yet unidentified proteins (C. Sucharov, A. Basu, and N. Avadhani, unpublished), whereas the two subsequent clusters (+17–22 and +24–29) map to sequences that bind to the heterodimeric GABP transcription factor described previously (5,19,36,40,41).

Affinities of the COX Vb Downstream ets Sequence Repeat for GABP Factor Binding

The GABP binding affinities of the two ets motifs from the COX Vb promoter corresponding to the +18 to +26 initiation region were studied using the +12 to +31 (ets-ets) double-stranded DNA probe. Bacterially expressed purified GABP α and β proteins or nuclear extracts from mouse 3T3 cells were used for comparison. As shown in Fig. 3, addition of GABP α protein to the wild-type ets-ets DNA probe yielded a single band (α1 complex), suggesting a single site binding (lane 1). Addition of α plus β yielded slower migrating complex (lane 2), consistent with a heterodimeric $\alpha1\beta1$ complex that is distinct from the $\alpha2\beta2$ complex (7) formed with the COX IV +5/+35 probe containing two high-affinity GABP binding sites (latter results not shown). A similarly migrating complex is detected with the nuclear extract from 3T3 cells (lane 6). A weak, slow migrating complex with the bacterially expressed α and β proteins marked as 2AB probably represents a tetramer ($\alpha2\beta2$), although its precise nature currently remains unknown. Results in Fig. 3 also show that ets-mut DNA (lanes 3 and 7) and mut-mut DNA (lanes 5 and 9) probes show negligible protein binding activity with both purified α and β subunits and also 3T3 nuclear extract. The mut-ets probe, on the other hand, yields significant, but reduced, $\alpha1\beta1$ complex with both purified α and β factors and 3T3 nuclear extract (lanes 4 and 8). This probe also formed a yet uncharacterized complex with 3T3 nuclear extract, but not with purified factors that comigrated with the α1 complex. In support of the relative factor binding affinities of the mutant probes, competition experiments (not presented) showed that mut-ets DNA effectively competes for GABP complex formation with et-ets DNA probe, whereas ets-mut DNA competes very weakly. Consistent with the results of Brown and McKnight (5) on the se-

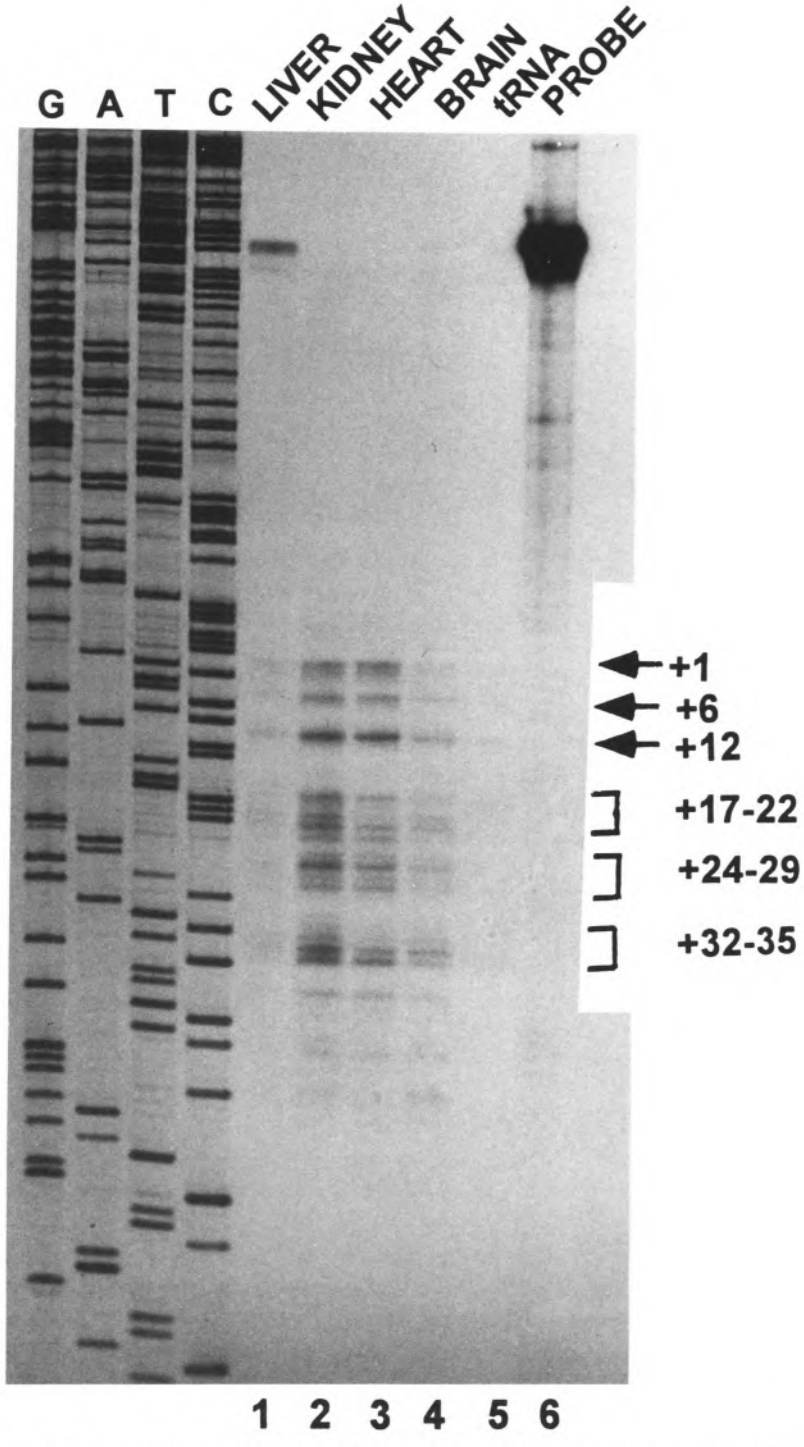


FIG. 1. Heterogeneous 5′ ends of COX Vb mRNA from different mouse tissues. S1 nuclease protection was carried out using 100 μg RNA from liver (lane 1), kidney (lane 2), heart (lane 3), brain (lane 4), or *E. coli* tRNA (lane 5) as indicated. In lane 6 undigested probe (50,000 cpm) was loaded. The details of S1 analysis and probe preparation were as described in the Materials and Methods section. The sequencing ladder was generated using the same primer that was used to prepare the S1 single-stranded DNA probe, and the COX Vb genomic subclone as the template.

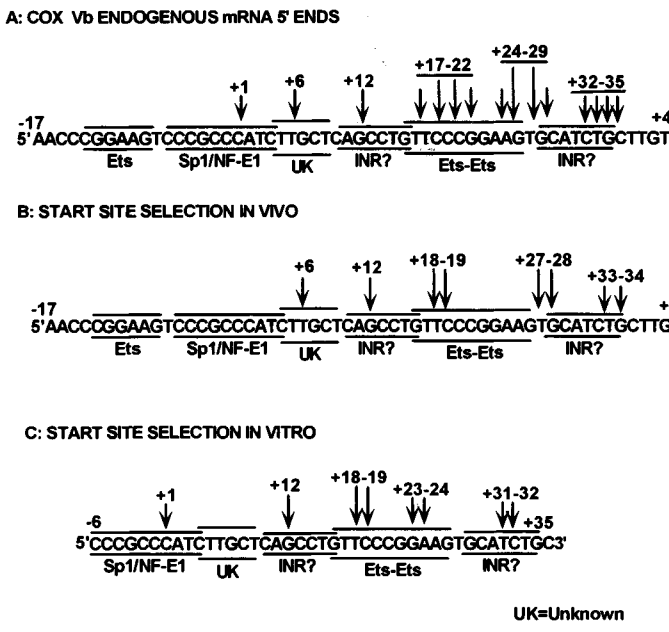


FIG. 2. Comparison of endogenous COX Vb mRNA 5′ ends with the transcription start sites selected under in vivo and in vitro conditions. The endogenous COX Vb mRNA 5′ map positions shown in (A) were based on the S1 nuclease analysis presented in Fig. 1. The nucleotide sequence of −17CAT DNA and various protein binding motifs are shown. (B) The 5′ terminal positions of RNA from COS cells transfected with −17CAT, −6CAT, and ets-ets constructs, and assayed by S1 nuclease protection presented in Fig. 6. (C) Transcription start site selection under in vitro transcription conditions using −6CAT (−13 to +35) and ets-ets (+12 to +31) templates as determined in Fig. 6B and C. The nucleotide sequence of the −6CAT DNA is presented.

quence requirements for GABP binding, the first ets motif with the GGGAAC sequence has a very weak affinity for GABP factors whereas the downstream sequence CGGAAG has a high affinity.

Functional Characterization of the GABP Binding ets Sites From the Transcription Initiation Region

The role of the GABP factor binding sites in transcription initiation or positioning was studied by in vivo expression of 5′ deletion CAT constructs or synthetic DNAs cloned in the pCAT basic plasmid. The level of expression and the start site positioning were assayed by CAT activity measurements in transfected 3T3 cells and S1 nuclease analysis of expressed RNA in COS cells, respectively. As shown in Fig. 4, the −17CAT DNA containing both intact upstream and downstream ets sites, as well as the Sp1/YY-1 sites, yielded maximum transcription stimulation (lane 1) whereas the 3′ as well as 5′ truncated −6CAT construct (sequence −6 to +35) yielded 53% activity (lane 2). Deletion of the Sp1/YY-1 sequence (sequence +12 to +31, ets-ets-CAT construct) further reduced activity to about 33–35% of −17CAT (lane 3), confirming the importance of this region in COX Vb promoter function. Mutation of the first of the two IR ets sequence repeats (Mut-ets-CAT) located on the transcribed strand (+16 to +21) did not affect the transcriptional activity (lane 5); however, mutations in the second ets site (ets-mut-CAT) from the sense strand (+22 to +25) reduced the activity to about 10–12% (lane 4). Finally, the construct mut-mut-CAT, carrying mutations in both of the ets sites, yielded significant, albeit low activity, possibly reflecting the activity of the protein binding motif at the +12 site (lane 6). Introduction of an Sp1 site 15 nucleotides upstream of the ets-ets motif (Sp1/ets-ets, lane 7) at a position equidistance to that present in the −17CAT DNA resulted in a near twofold higher activity. The Sp1 stimulation of activity appears to be distance dependent because an Sp1 site placed at 56 nucleotides upstream (2XSp1/ets-ets) had no effect on the transcription activity of the ets repeat motif (lane 8). As expected, pCATb yielded marginal activity in the range of 3–4%.

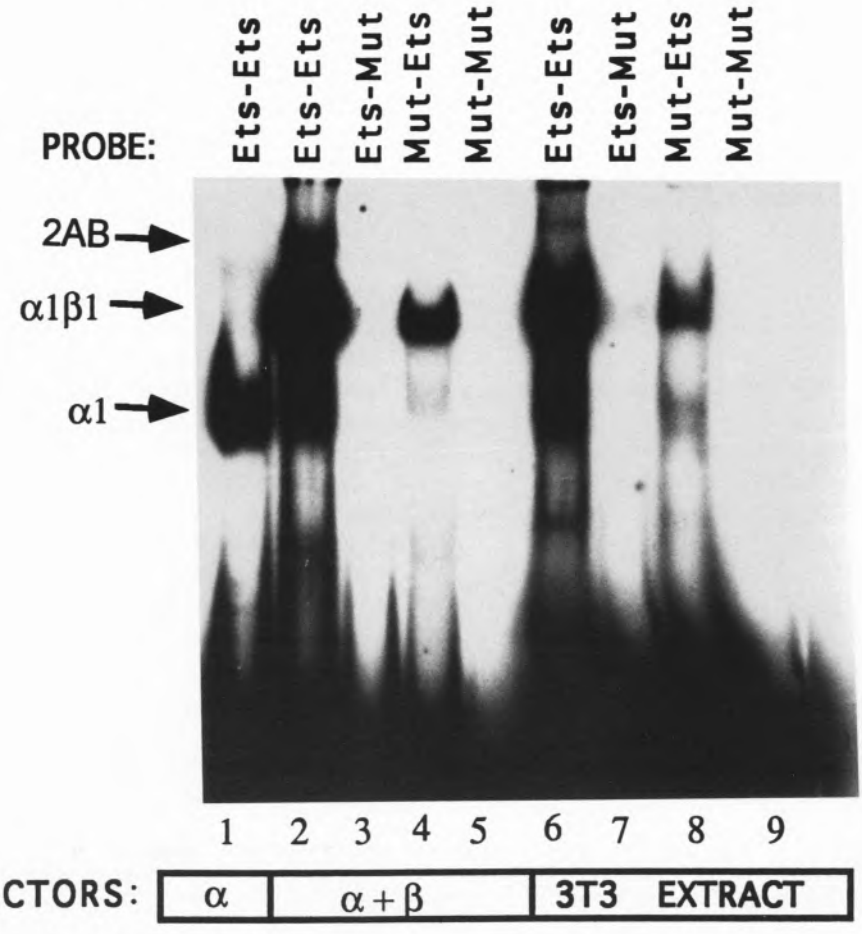


FIG. 3. Protein binding profiles of the GABP binding ets repeat motif from the COX Vb gene. ets-ets (lanes 2 and 6), ets-Mut (lane 3 and 7), mut-ets (lanes 4 and 8), and mut-mut (lanes 5 and 9) double-stranded DNA were 5′ end labeled and assayed for protein binding by gel mobility shift analysis as described in the Materials and Methods section. Bacterially purified (100 ng) α subunit (lane 1), 100 ng α plus 500 ng of β subunit (lanes 2–5), and 5 μg of 3T3 nuclear extract (lanes 6–9) were used as indicated at the bottom of the figure. Protein–DNA complexes with α alone (α1), $\alpha 1\beta 1$ heterodimer, and 2AB (a possible tetramer) are indicated with arrows.

In a recent study we showed that the +5 to +35 region of the COX IV promoter containing both of the GABP binding ets sites can initiate transcription under both in vivo and in vitro conditions (7). In the present study, we sought additional evidence on the role of GABP factors on the activities of both COX IV and Vb minimal ets promoters by coexpression with cDNAs encoding GABP α and β subunits. Overexpression of GABP α in the absence or presence of β in 3T3 cells produced no significant change in activity driven by COX IV WT reporter with both ets sites intact (Fig. 5A). However, cotransfection with β cDNA alone resulted in a modest (50%) increase in activity of the WT reporter. Similarly, coexpression of GABP α alone had no effect on the activity of the COX IV Mut1 construct with a single ets site, although coexpression of β alone or α plus β caused a threefold stimulation of activity (Fig. 5A). Coexpression of GABP α and β in both COS and 3T3 cells failed to stimulate the activity of the COX IV WT reporter. In contrast, the activities of both COX IV and Vb single ets site promoters, Mut1 and mut-ets-CAT, respectively, were stimulated two- to threefold by coexpression with GABP α and β cDNAs (Fig. 5C).

The transcriptional start sites under in vivo conditions were determined by S1 nuclease protection of COS cell RNA transfected with various COX Vb promoter constructs. The −17CAT DNA yielded four discrete transcripts with 5′ termini mapping to +6, +12, +18–19, +27–28, and +33–34 (Fig. 6A). The 5′ map positions of RNA from cells transfected with the 17CAT DNA

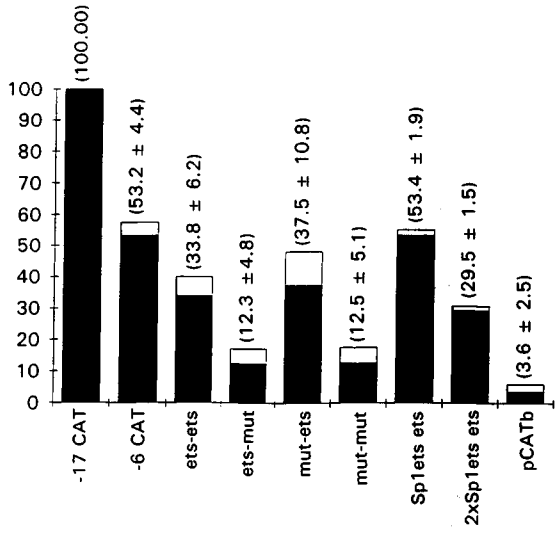


FIG. 4. Transcriptional activities of COX Vb basal promoter constructs by transient expression in 3T3 cells. CAT plasmid DNA constructs as indicated were transfected in 3T3 cells along with the reporter plasmid SV-40-βgal and assayed for CAT activity as described in the Materials and Methods section. The nucleotide sequence of −17CAT DNA is presented in Fig. 2, and all other basal promoter sequences are presented in the Materials and Methods section. The standard deviations were calculated from three independent transfections. Numbers in parentheses indicate the actual CAT values.

resembled the major species of protection obtained with endogenous RNA from mouse tissues, excepting that the transcript mapping to the +1 position is missing (Figs. 2 and 6A). The −6CAT construct (Fig. 6B), which lacks the upstream GABP consensus motif at −13 to −8 position, yielded RNA with identical 5′ ends as the −17CAT construct, implying that the upstream GABP site may not have a role in transcription start site positioning. The ets-ets-CAT construct containing the +12 to +31 sequence yielded transcripts mapping to +12, +18–19, and +27–28 positions, and lacked transcripts mapping to +6 and +33–34 positions. These results suggest that the *cis* DNA elements required for the initiation of transcription at +12, +18–19, and +27–28 positions are contained within the +12 to +31 region of the promoter. In all of the above cases, RNA from control untransfected COS cells failed to show protection corresponding to these positions (see control RNA in Fig. 6A–C).

To determine the relative importance of the upstream (antisense strand) and downstream (sense strand) ets sites on transcription initiation, CAT constructs containing mutated ets sites were expressed in COS cells and the RNA was used for S1 mapping. As shown in Fig. 7A, the RNA from cells transfected with mut-ets-CAT DNA gave marginal protection below the threshold level for detection (lane 2), whereas RNA from cells cotransfected with GABP α and β cDNAs along with the mut-ets-CAT reporter (lane 1) yielded distinct protected fragments mapping to +20–22 and +26–29 positions. These results suggest that mutations at the ets site with low-affinity GABP binding consensus results in a shift of both clusters of initiation by 3–4 nucleotides downstream. The S1 pattern in lane 1, however, showed that an overexpression of GABP α and β markedly reduced transcripts mapping to the +12 position. The mechanism by which a high level of GABP binding to the ets motif might inhibit transcription at the +12 position remains unclear, although it may involve stearic hinderance or competition for factor binding to very closely positioned sequence motifs as described later in this section. Additionally, results in Fig. 7A show that ets-mut reporter with or without coexpression with the α and β cDNAs failed to show any detectable protection (lanes 3 and 4).

An in vitro transcription system derived from HeLa cells was used to further investigate the transcription initiation sites and activities of various COX Vb mutant constructs. As seen from Fig. 7B, mut-ets-CAT template yielded in vitro transcripts starting at positions +12, +21–22, and +26, whereas both ets-mut-CAT and mut-mut-CAT templates yielded transcripts lacking the +21–22 and +26 clusters. These results suggest that the sense strand ets site with a strong consensus for GABP factor binding plays an important role in transcription initiation whereas mutations at the upstream site causes a shift in the positioning of the transcripts under both in vivo and in vitro conditions.

GABP Factor Dependence of Transcription Initiation at the COX Vb and COX IV ets Motifs

The role of the GABP factors in transcription initiation at the +18–19 and +23–24 sites was investigated by in vitro transcription in HeLa nuclear extracts depleted of the α and β subunits either by immunoadsorption or adsorption to +16–27 minimal ets-ets DNA affinity matrix. Gel mobility patterns in Fig. 8 show the effects of GABP α and β depletion by affinity adsorption to DNA–Sepharose resin. As shown in lane 2, nuclear extract incubated with ets-ets DNA–Sepharose exhibited negligible complex formation with the ets-ets DNA probe corresponding to complexes obtained with bacterially expressed/purified GABP α alone (lane 5) and α + β hetero-

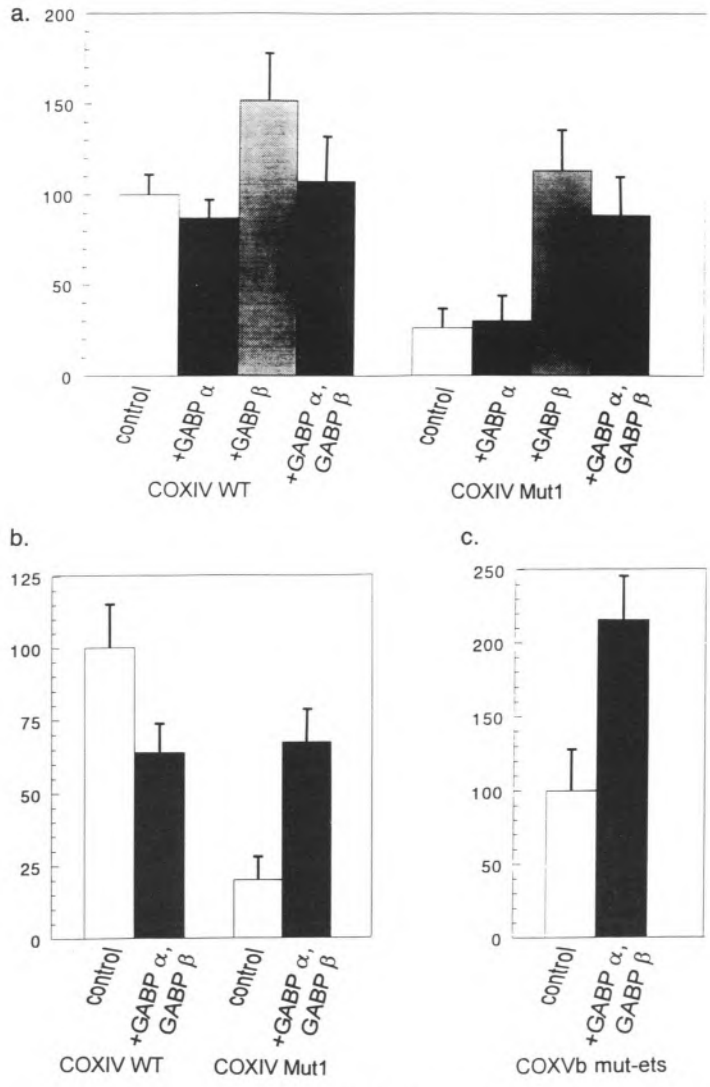


FIG. 5. Stimulation of COX IV and Vb IR promoter activity cotransfected CMV/GABP expression plasmids. Transfections of COS (A) and 3T3 (B, C) cells were performed with either the COX IV (+5/37) WT or Mut1 reporter plasmids or COX Vb mut-ets plasmids with indicated GABP α and/or β (1 μg each) CMV promoter-driven plsmids. Relative CAT activities based on the protein contents of the extracts were normalized to the activity of the WT or mut-ets reporter plasmids in control transfections without CMV/GABP expression plasmids. The use of β-gal reporter plasmids was avoided in these coexpression experiments because of its squelching effects on the expression of CMV-based GABP plasmids. Average and standard deviations were calculated from four to six transfections.

dimer (lane 6). The extract treated with ets-mut DNA–Sepharose, on the other hand, yielded a prominent band corresponding to the α and β heterodimer, designated as $\alpha 1\beta 1$ complex (lane 3). A lower intensity of the $\alpha 1\beta 1$ complex and the lack of significant α-bound complex (designated as $\alpha 1$) in lane 3 compared to the control extract in lane 1 is probably due to a two- to threefold increase in volume of the protein extract during repeated adsorption to the affinity resin and subsequent dialysis. Similarly, protein purified by binding to the ets-ets resin showed both $\alpha 1$ and $\alpha 1\beta 1$ complexes (Fig. 8, lane 4). Although not shown, a similar fraction eluted from the ets-mut DNA-bound resin did not show any binding. Additionally, the extract subjected to ets-ets-DNA–Sepharose binding yielded two prominent complexes with the COX Vb 17Inr probe, which contains an Sp1 motif fused to a YY-1 sequence motif (2). Although not shown, the identity of Sp1 and YY-1 (NF-E1)

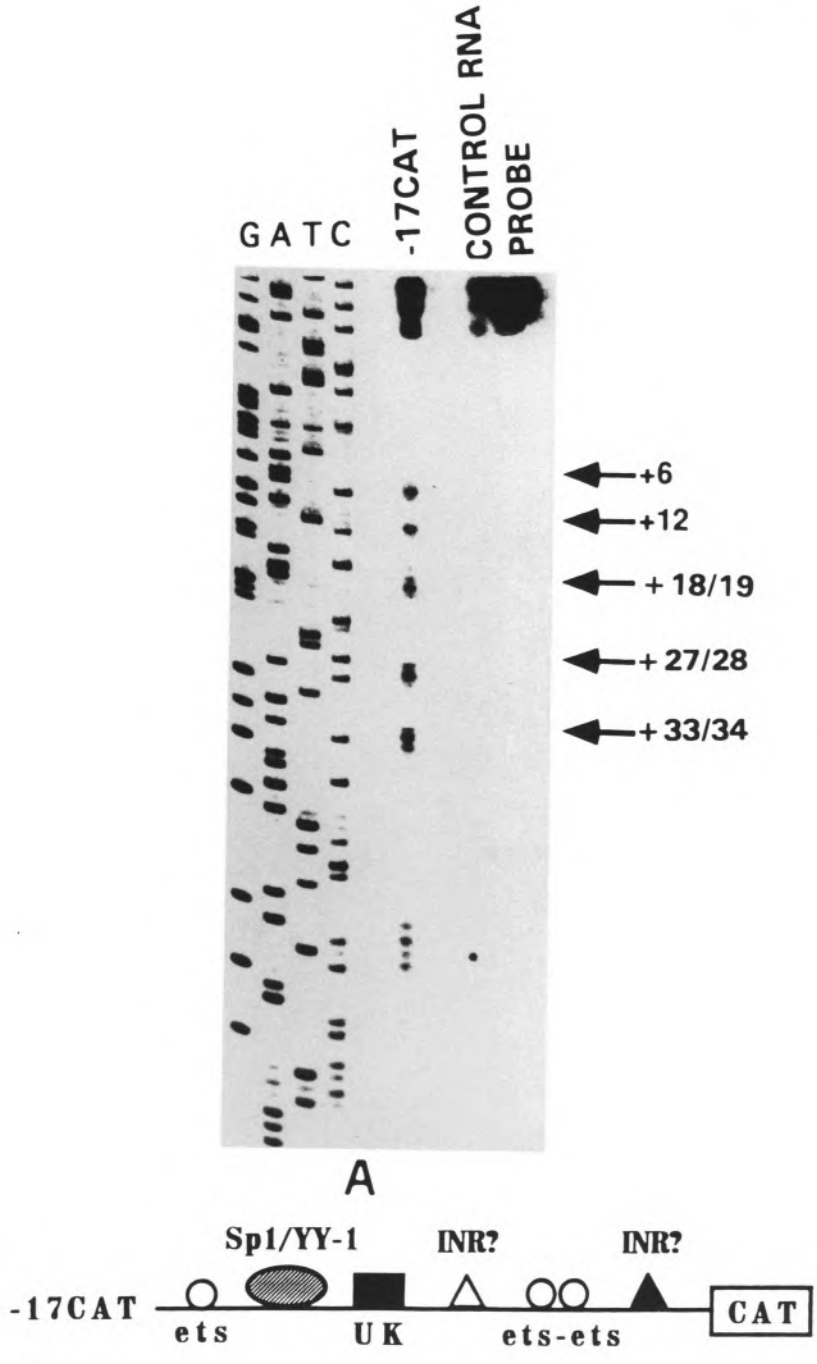
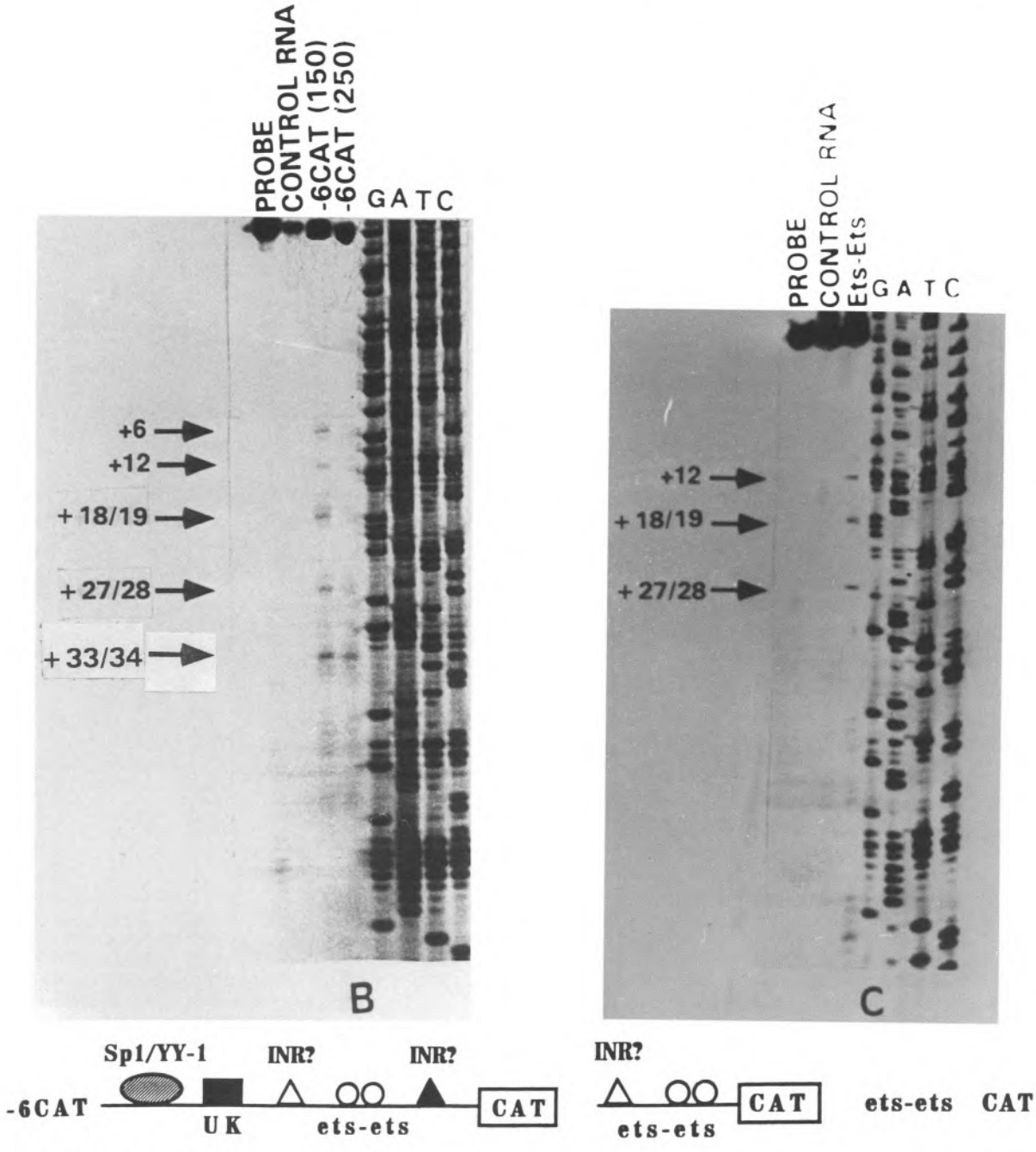


FIG. 6A.

bound complexes were based on competition with sequence-specific DNA motifs described previously (2). These results suggest that the affinity adsorption leads to a selective depletion of α and β factors from the HeLa nuclear extract.

Results in Fig. 9A shows that -6CAT template in untreated HeLa nuclear extract yielded four major transcripts starting at $+1$, $+12$, $+18$–19, and $+23$–24 positions in addition to minor transcripts at $+14$ to $+16$ and $+31$–32 regions (lane 1). The in vitro products, however, lacked detectable transcripts mapping to the $+6$ position that were observed under the in vivo conditions. At present, the reasons for the apparent absence of these transcripts under in vitro conditions remain unknown. Extracts depleted of GABP α and β



ABOVE AND FACING PAGE

FIG. 6. Transcriptional start site selection on the COX Vb basal promoter constructs under in vivo conditions. Various basal promoter elements cloned in the pCATb plasmid vector were transfected in COS cells as described in the Materials and Methods section. About 48 h after transfection, total RNA was isolated and used for S1 analysis using the appropriate single-stranded DNA probes prepared by the Klenow extension of the CAT-reverse primer. (A) The S1 nuclease protection of the −17CAT probe with RNA from cells transfected with the −17CAT DNA and RNA from untransfected COS cells. S1 (150 units) was used in each case. (B) S1 protection of the −6CAT DNA probe control COS cell RNA and RNA from cells transfected with the −6CAT DNA. The numbers, 150 and 250, in parentheses indicate the amount of S1 nuclease used for each digestion. (C) The S1 protection pattern of ets-ets probe with control COS cell RNA and RNA from cells transfected with the ets-ets plasmid DNA construct. S1 (150 units) was used in each case. The nature of CAT constructs used for transfection and S1 protection in each case has been illustrated at the bottom of figures. The sequencing ladders were generated with the appropriate template DNAs indicated and the CAT-reverse primer.

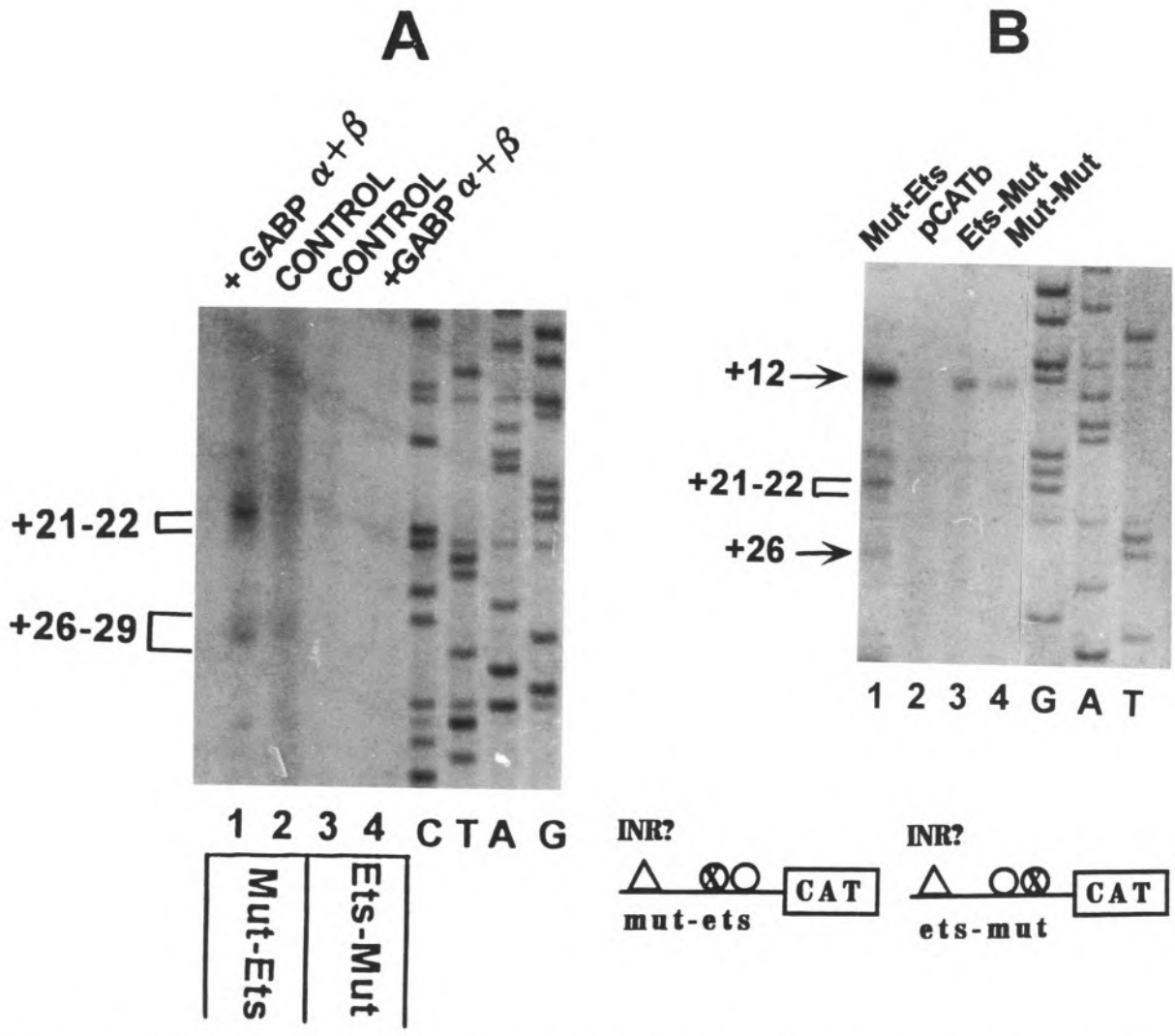


FIG. 7. Downshifting of transcription start sites under both in vivo and in vitro conditions by mutations at the upstream ets site of COX Vb IR construct. (A) Mut-ets (lanes 1 and 2) or ets-Mut (lanes 3 and 4) were transfected in COS cells with or without coexpressing GABP α + β cDNAs, and RNA (100 μg each) from these cells were assayed by the S1 nuclease protection as described in Fig. 6 and in the Materials and Methods section. (B) Various mutant DNA templates (450 ng supercoiled DNA/16 μl reaction volume) were assayed for in vitro transcription activity using the HeLa nuclear extract as described in the Materials and Methods section. The nature of the mut-ets-CAT and ets-mut-CAT templates used is illustrated at the bottom of the figures.

factors by DNA affinity adsorption (lane 3) or immunodepletion (lane 4) failed to support transcription initiation at +18–19 and +23–24, as well as minor species at +14–16 sites, although both treated extracts supported transcription initiation starting at +1, +12, and +31–32 positions (lanes 3 and 4). In repeated attempts, extracts depleted of GABP factors showed relatively higher level of transcripts originating at +12 and +32–33 positions, possibly due to reduced stearic hinderance for factor binding to these positions or reduced competition for binding basal transcription factors (C. Sucharov and N. Avadhani, unpublished results). Results in lane 2 also showed that an extract treated with ets-mut DNA–Sepharose, containing both α and β factors (lane 2), initiated transcription starting at all of the major as well as minor sites, similar to the control untreated extract shown in lane 1. Additionally, transcription activity of another unrelated Inr construct, (G6TI) containing an SV40 early GC boxes with downstream TATA box and the AdML Inr (24), was unaffected when extracts were depleted of α and β by either of the procedures (Fig. 9B).

In Fig. 10, COX Vb ets-ets minimal promoter construct (+12 to +31), which lacks the +12–16 and +31–35 protein binding motifs, was used as a

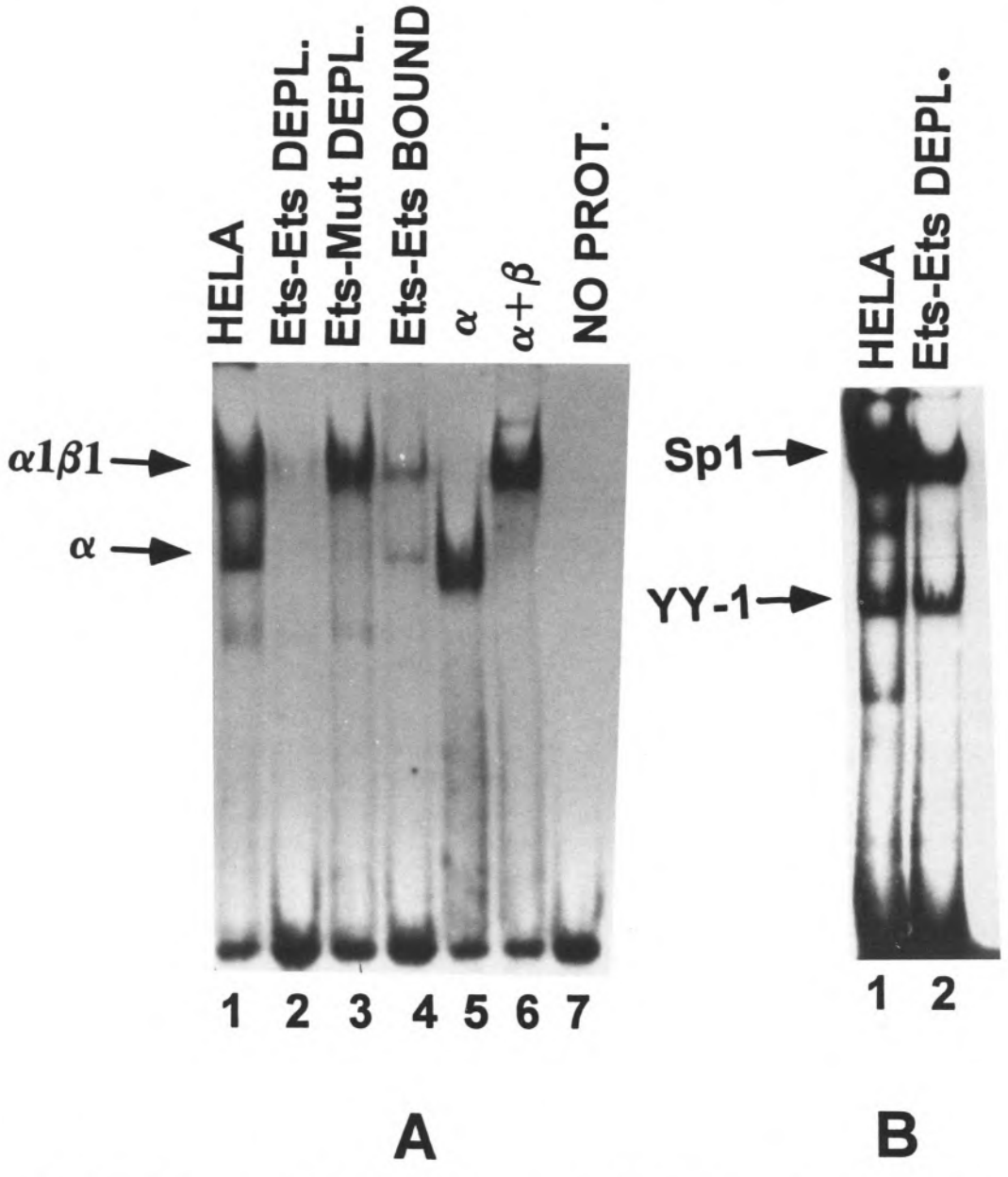


FIG. 8. DNA binding patterns of the HeLa nuclear extract depleted of GABP factors by adsorption to DNA affinity resin. Nuclear extracts were treated three times with ets-ets DNA–Sepharose or ets-Mut DNA–Sepharose, and the bound GABP factors with the ets-ets resin were purified by elution with high-salt buffers as described (19,41). The extent of GABP factor depletion and also the binding patterns of the affinity-purified factors were tested by gel mobility shift analysis using ^{32}P-labeled ets-ets DNA probe (A) and COX Vb 17Inr DNA probe (B). Untreated HeLa nuclear extract (4 μl each) or extracts adsorbed to ets-ets DNA resin (ets-ets depleted) or ets-Mut DNA resin (ets-Mut depleted) were used for DNA binding as described under the Materials and Methods section. HeLa nuclear GABP α and β (1 μl; about 3–5 ng protein) and 100 ng of bacterially expressed α and 500 ng of bacterially expressed β proteins were used. The relative migration of DNA-bound α and heterodimeric $\alpha 1\beta 1$ complexes with the ets-ets probe, and also Sp1- and YY-1-bound complexes with the 17Inr probe are indicated.

template for in vitro transcription in GABP-depleted and replenished extracts. In support of the possible stearic hinderance for protein binding to sequence motifs closely positioned within the +12 and +35 region of the promoter, the minimal ets-ets-CAT DNA template (+16 to +27) consistently yielded several-fold higher levels of transcripts mapping to both +18–19 and +23–26 ets sites compared to the − 6CAT template (Figs. 9A and 10A). This minimal ets-ets-CAT template yielded major transcripts originating at +18–19 and +23–26 positions in untreated extracts,

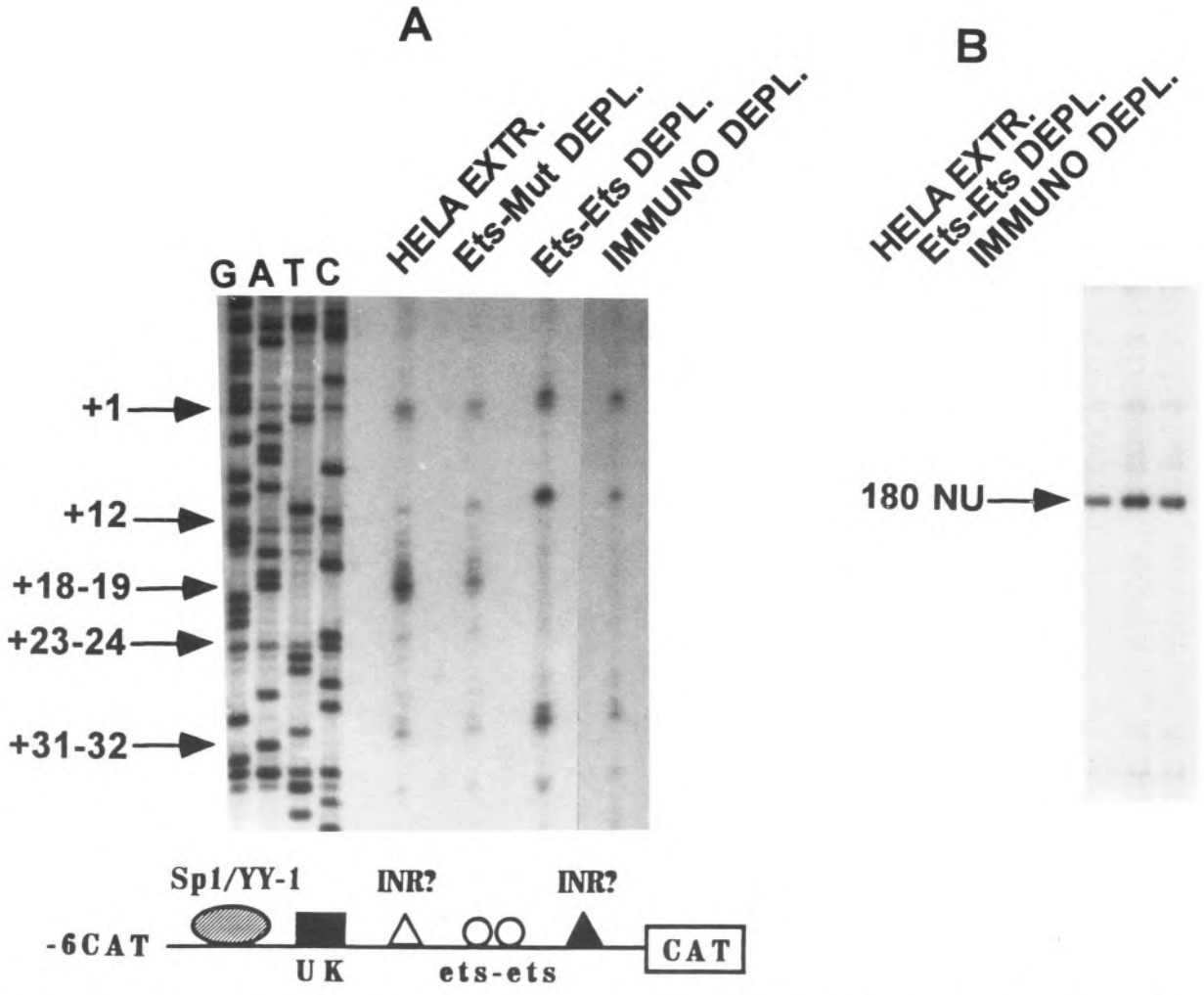


FIG. 9. Effects of GABP α and β subunit depletion on the in vitro transcriptional activity. HeLa nuclear extracts were depleted of GABP α and β subunits either by immunodepletion or DNA affinity adsorption described in the Materials and Methods section. Although not shown, the immunodepletion was nearly complete as tested by gel mobility shift assays. (A) 450 ng of -6CAT template (sequence -6 to $+35$, illustrated at the bottom) was used in each assay. In vitro transcription was carried out in 16-μl reaction volumes and the transcription start sites were detected by primer extension of ^{32}P-labeled CAT-reverse primer as described in the Materials and Methods section. (B) 400 ng per assay of G6Ti promoter construct was used as the template. T7 primer was used for the reverse transcriptase-based detection of transcription start site.

whereas both immunodepleted and ets-ets DNA affinity-adsorbed extracts failed to support transcription originating from these positions. The extract adsorbed to weak binding ets-mut DNA matrix, on the other hand, yielded almost all the species obtained with the untreated extract. The lower intensities of bands in this lane may be due to the dilution effect described previously. The results also show that GABP α and β subunits purified by DNA affinity binding of HeLa nuclear extract fully restored transcription initiation at $+18$–19 and $+24$–26 positions in both immunodepleted and affinity DNA-adsorbed extracts. Additionally, bacterially expressed, purified GABP α and β proteins also restored activity of the DNA affinity-depleted extract. As shown in a recent study from this laboratory (7) and also in Fig. 10B (lane 1), the COX IV WT DNA template ($+5$–35 sequence) containing both ets sites supported in vitro transcription mainly starting from the downstream (DS) ets site. As observed with COX Vb minimal ets-ets promoter, both immunodepletion and DNA affinity adsorption abolished transcription at this site. The results also show that addition of either bacterially expressed GABP α and β proteins or those purified from HeLa nuclear extract restored transcriptional activity.

DISCUSSION

Recent studies in our laboratory showed that basal promoter activities of the TATA-less genes

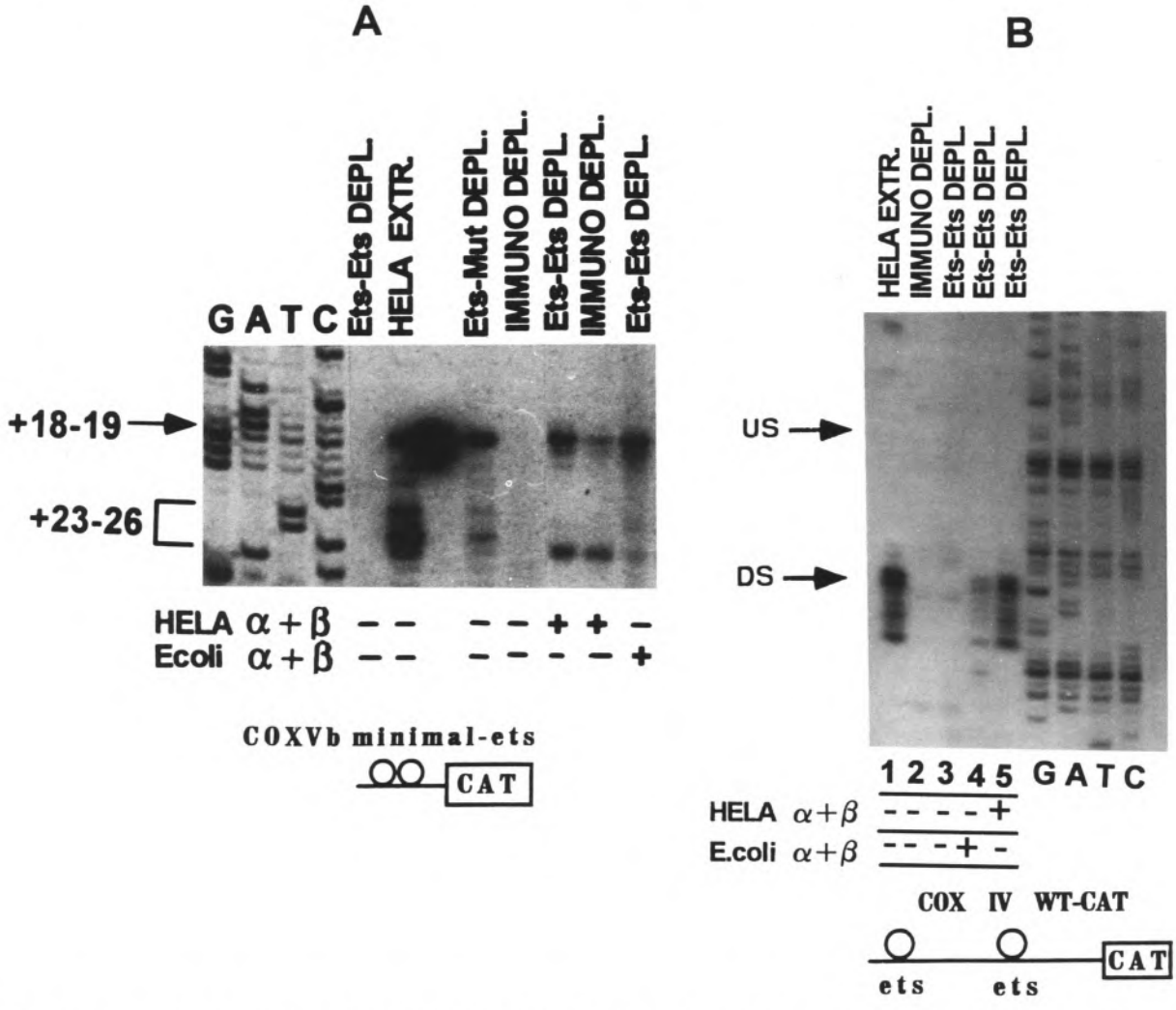


FIG. 10. Restoration of transcription initiation activity of the depleted HeLa extract by purified GABP α and β subunits. In vitro transcription assays were carried out using the COX Vb ets-ets-CAT DNA template (A) or COX IV WT template DNA (B). For reconstitution of activity, bacterially expressed and purified α (200 ng) plus β (500 ng) or 5–8 ng of DNA affinity-purified $\alpha + \beta$ from HeLa nuclear extract were used. Other details were as in Fig. 9 and in the Materials and Methods section. The respective template DNAs used for in vitro transcription are illustrated at the bottom of each figure.

encoding the murine cytochrome oxidase (COX) subunit IV and Vb are dependent on sequences surrounding the multiple sites of transcription initiation, in addition to upstream Sp1 sites (2,7,8). Both COX IV and Vb genes contain multiple CGGAAG motifs that bind to the heterodimeric transcription factor GABP, consisting of an ets family DNA binding α subunit and a non-DNA binding β subunit (5,36). Functional studies showed that the GABP factor binding sites are important for the transcriptional activities of both COX IV and Vb promoters (2,7,8). Our results also showed that a 21-bp tandem repeat sequence from the transcription initiation region of the COX IV promoter, containing the GABP factor binding motifs, can activate transcription in the absence of other flanking sequences (7). In addition to a YY-1 (NF-E1) motif around the +1 position, the COX Vb basal promoter contains a duplicated ets sequence motif at positions +16 to +26. Results presented in this study demonstrate that the COX Vb and COX IV ets sequences function as an initiator under both in vitro and in vivo conditions and that the activities of the IR ets sequence repeats under in vitro conditions are dependent upon the presence of GABP α and β subunits. It is known that Sp1 factor binding motif in promoter constructs lacking both TATA and consensus Inr can activate transcription originating at nonspecific or cryptic vector sites (6,33). A detailed comparison of in vivo and in vitro transcription initiation sites in the present study, however,

demonstrated that the ets initiator activates transcription at specific sites rather than activating cryptic sites.

Transcription initiation mapping to the +1 position of the COX Vb promoter was previously shown to depend on a Inr-like YY-1 (NF-E1) factor binding motif around this region (2). Transcripts starting at +6 position map to a sequence binding to an as yet uncharacterized protein (data not shown). The sequences around RNA start sites at +12 and +32–35 (CAGCCTG and CATCTG) are analogous to sequences described as "weak Inrs" (16). The Inr-like motifs at +12–16 and +31–35 bind to yet uncharacterized protein from HeLa nuclear extract (C. Sucharov, A. Basu, and N. G. Avadhani, unpublished) and, additionally, our results show that binding of GABP to the +16 to +26 position affects the extent of factor binding to the +11 to +16 and also +31 to +35 positions. This apparent stearic hinderance between protein factors binding to the adjacently located sequence motifs is consistent with the in vitro transcription data (Figs. 9 and 10) showing higher levels of transcripts starting at the +12 and +32–34 in extracts depleted of GABP α and β, and also increased initiations at the +23–26 site with DNA templates lacking the +31–35 sequence motif. Thus, although the nature of protein factors binding to some of these motifs and also their functional roles remain unknown, it is interesting that the COX Vb mRNA 5′ ends map to a cluster of different protein binding sites that are organized in a close proximity to each other with very limited spacer regions in between.

Transient expression of −17CAT or −6CAT constructs containing all of the intact protein binding sites downstream as well as immediate upstream of +1 yielded S1 patterns essentially similar to the endogenous RNA (Figs. 2 and 6), excepting that they lack RNA starting at +1 position. As reported previously (2), the YY-1 motif responsible for the +1 positioning on the COX Vb promoter overlaps a GC box and binds YY-1 (NF-E1) or Sp1 exclusive of each other. We believe that the absence of the +1 transcript in the in vivo-expressed RNA reflects the high level of Sp1 relative to YY-1 (NF-E1) in COS cells. Identical transcription start sites at +12 and +18–19 positions are seen under in vivo conditions as determined by S1 mapping, and also in an in vitro transcription system (Fig. 2B, C). The in vitro and in vivo experimental conditions also show some notable differences with respect to transcriptional start sites. 1) Transcription initiation at +6 position observed under in vivo transient expression conditions and also by S1 mapping with endogenous mRNA is not observed under the in vitro conditions (Figs. 2, 6, and 9). Reasons for the absence of +6 transcripts under in vitro conditions remain unknown, although the inefficiency of the in vitro system or possible inactivation of some essential protein factors are likely possibilities. 2) There is a significant shift in the positioning of transcripts mapping to the downstream ets site from +27–28 under in vivo conditions to +23–24 under in vitro conditions (Figs. 2, 6, and 9). Furthermore, the position of the downstream cluster (+23–27) shows a significant variation depending on whether the template DNA contains an intact protein binding site at +31–35, and also the relative GABP factor concentration. Use of a minimal et-ets-CAT template and reconstitution with purified α and β subunits shift the downstream start site more towards the position similar to that observed under in vivo transient expression conditions (Figs. 6 and 10A). 3) The position of transcripts that require the presence of the +31–35 motif vary from +31–32 under in vitro conditions to +33–34 under in vivo conditions. Additionally, transcripts mapping to the +31 to +35 position are expressed as minor components in an in vitro system whereas they are expressed as major components under in vivo transient expression conditions. Conversely, transcripts mapping to the ets site at the +18–19 position are expressed weakly under in vivo but very prominently under in vitro conditions. Despite these inherent differences, transcripts mapping to the +18–19 (upstream ets) site and a more heterogeneous/variable cluster mapping to the +23–29 position (downstream ets site) are expressed in both of the systems.

The −6CAT construct lacking the upstream ets site at −13 to −8 exhibits about 50% less CAT activity compared to the −17CAT construct with an intact ets site at this position (Fig. 4). The results of S1 nuclease analysis, on the other hand, show no qualitative difference with respect to transcription start sites (Fig. 6). Additionally, the −6CAT template lacking the upstream ets sequence can efficiently initiate transcription at +1 as well as other downstream sites under in vitro conditions. These results suggest that the GABP binding motif located upstream of +1 (at −13 to −8) may not have any direct role in positioning of transcription initiation. Nevertheless, it may have an important activation or enhancer function similar to that shown for GABP binding sites at upstream positions of a number of other genes (12,30,38,39,42). The downstream ets sequence re-

peat at +16 to +26, on the other hand, appears to act as a transcription initiator by multiple criteria. 1) A minimal promoter consisting of the COX Vb ets sequence repeat exhibits 30–35% of CAT activity compared to −17CAT DNA (Fig. 4). Mutations at the downstream site positioned on the sense strand and also mutations to both sites yield marginal activity in the range of 8–12% of the activity obtained with the −17CAT DNA. 2) The minimal promoter with the COX Vb ets sequence repeat can initiate transcripts mapping to +18–19 and +23–26 positions under both in vivo and in vitro conditions. Mutations at the downstream ets site, which exhibits a higher affinity for GABP binding, or both sites, abolished or reduce the ets motif specific transcripts mapping to the +18–19 and +23–27 sites under both in vivo (Fig. 7) and in vitro conditions (Fig. 7). 3) An Sp1 motif placed at 15 nucleotides upstream of the COX Vb ets repeat motif caused a near twofold increase of transcription from the ets motif positioned start site. A similar activation role was shown for upstream TATA or Sp1 sites for the pyrimidine-rich PyCAPy initiators (33,34,45).

In vitro transcription experiments using the wild-type and mutant templates support the view that the initiator activity of COX IV and COX Vb IR ets sequence are dependent on the GABP α and β factors. Extracts depleted of GABP α and β, using specific antibodies or DNA affinity adsorption, fail to support transcription initiation at the COX IV and Vb ets-specific sites (Fig. 10A, B). The loss of activity appears to be specific for ets repeat initiators because initiation at +1 position of the promoter that was previously shown to depend upon the presence of multifunctional transcription factor YY-1 (NF-E1) is not affected (Fig. 9A). Similarly, efficient transcription initiation at +12 and +31–32 positions of the promoter are observed in extracts depleted of GABP (Fig. 9A). Additionally, initiation with the TdT-derived Inr of the G6TI promoter (24) was unaffected by the depletion (Fig. 9B). Thus, our results that factor depletion by two different approaches do not affect the function of three other COX Vb IR motifs with transcription initiator activities, nor the Inr of the G6TI promoter, point to the high degree of specificity of factor depletion. The requirement of GABP α and β subunits for the ets initiator function is demonstrated by the observation that bacterially expressed, purified subunits can fully restore the activities of depleted extracts (Fig. 10). Transcriptional reconstitution required 50- to 100-fold higher molar concentrations of bacterially expressed GABP factors compared to the HeLa cell-purified factors. Currently, the reasons for this large difference in the activities of GABP factors purified from these two sources remain unclear.

Coexpression of GABP α and β cDNAs in both COS and 3T3 cells shows different effects on the activities of COX IV IR promoters containing both intact (WT) and single ets site (Mut1), possibly reflecting their relative factor binding efficiencies. A previous study from our laboratory showed that the efficiency of GABP factor (α and β subunits) binding to the COX IV WT probe was 10- to 20-fold higher compared to the Mut1 probe with a single ets site (7). Thus, the inability of coexpressed GABP α plus β to stimulate transcription with the WT template suggests a possible saturating level of this factor in both COS and 3T3 cells. Stimulation of transcription with single ets site containing promoters by coexpression, on the other hand, reflects the low affinities of these motifs, which requires a higher steady-state levels of GABP factors for complex formation. Nevertheless, the results on two- to threefold stimulation of transcription activities of both single ets site promoters, Mut1 (COX IV), and mut-ets (COX Vb) by GABP α and β coexpression provide a direct support for the involvement of this factor in the ets-specific initiator function under in vivo conditions.

It is known that sequences containing the YY-1 (NF-E1) binding sites from the COX Vb and AdML P5+1 promoters (2,31) can function as transcription initiators. Our results showed that a minimal promoter containing the COX Vb YY-1 (NF-E1) motif was able to initiate transcription in vivo in 3T3 cells, but was unable to initiate transcription in *Drosophila* embryo extracts lacking YY-1. Addition of bacterially expressed YY-1 (NF-E1) reconstituted transcriptional activity in the *Drosophila* extract, demonstrating the dependence on this factor for transcription initiation at the +1 position. Evidence presented in this study demonstrates that the GABP factor binding ets sequence repeat from transcription initiation regions of two different COX genes functions as transcription initiator. It should be pointed out that the abolition of GABP factor binding to the COX Vb IR ets sequence (+16 to +26) does not affect the activities of initiators responsible for transcripts starting at +1, +12, and +31–33 positions. Hence, this GABP binding IR ets sequence repeat functions as a novel initiator rather than a global activator. Results presented in this study therefore support the view that in addition to the well-characterized PyCAPy initiator(s), a variety of the IR protein binding motifs from different

promoters can also function as transcription initiators. Many of these factor binding motifs, like the NF-E1/YY-1, GABP, E2F, etc., appear to be multifunctional in that they can function both as transcription activators and initiators, depending on their relative location within the promoter. These IR motif-bound transcription factors may directly or indirectly interact with TFIID or facilitate the formation of initiation complex by an unknown mechanism. In support of such a possibility, several gene-specific transcription factors, such as VP-16, CTF, NTF, Sp-1, USF, YY-1, Jun, Fos, EBV factor R, etc. (9,21,23-25,28,34,35, 37,44), have been shown to interact with TATA box binding protein or components of TFIID.

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