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A Single Element within the Hepatitis B Virus Enhancer Binds Multiple Proteins and Responds to Multiple Stimuli

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The hepatitis B virus enhancer can be dissected into multiple functional elements, one of which is the E element. We show here that the E element binds multiple nuclear proteins that are essential for its enhancer activity. These findings, together with the ability of this element to respond to at least two different viral transactivators, suggest that the E element is an enhancer modulator capable of binding different factors and responding to multiple stimuli.

The human hepatitis B virus (HBV) is a member of the *Hepadnaviridae*, a family of hepatotropic, enveloped animal viruses (for a review, see reference 6). One of the characteristic features of this family is the size of the DNA genome, which is very small (between 3.0 and 3.5 kilobases). This feature has strong implications for the genome organization, and at least 50% of the genome codes for two overlapping open reading frames. Accordingly, the viral transcription regulatory sequences, i.e., promoters and enhancers, are also embedded within an open reading frame. This unique character of the viral transcription regulatory sequences makes this virus an interesting model with which to investigate the basic components of promoters and enhancers. The HBV enhancer contains a sequence motif that is conserved in the genomes of most hepadnaviruses (2, 13). This motif, termed the E element, was defined by virtue of its capacity to bind nuclear proteins (12) and to function as an enhancer once it is multimerized (2). Interestingly, the enhancer activity of the multimer of the E element is induced by phorbol esters (2).

To investigate the enhancer activity of the E element, synthetic oligonucleotides corresponding to the E element were prepared and multimerized, and progressive copy numbers were cloned in the βe^- reporter plasmid. The activities of these plasmids were assayed in two different cell lines, Alexander and SK-Hepl (12). In agreement with our previous observation (2), the E element had a strong intrinsic enhancer activity in Alexander cells (Fig. 1, lanes 2 through 4). This activity correlated linearly with the number of copies of the E element, suggesting the absence of functional cooperativity between the E-binding proteins. A similar behavior was manifested in SK-Hepl cells; however, the E element acted very poorly (Fig. 1, lanes 6 through 9). The inefficient activity of the E element in SK-Hepl cells was further emphasized by the fact that a reference plasmid that contained the simian virus 40 enhancer and the α-globin promoter showed a strong transcription activity.

We next used the gel retardation analysis to explore nuclear proteins that bind to the E element (E proteins). The analysis was performed as described previously (2). A characteristic pattern of the E oligonucleotide in complex with nuclear proteins as resolved on a native gel is shown in Fig. 2, lane 2. At least five distinct complexes were obtained with nuclear extracts of Alexander cells. To determine the sequence specificity of these complexes, a set of mutants of the E element were prepared and analyzed. Mutants Em-3 and Em-4 bound proteins as well as the wild type (lanes 3 and 4); however, mutant Em-5 lost the capacity to bind proteins (lane 5). This suggests that the sequence AC within the E element, which was modified in the mutant Em-5, is crucial for the formation of DNA-protein complexes. Nuclear extracts of SK-Hepl cells basically formed similar complexes with the E element, with a similar sequence specificity, but this cell line was much less abundant in E proteins (lanes 7 through 10). As a control, we compared the amount of the EP protein, which also binds the HBV enhancer but at a different site (2), in these cells and found it to be similar (compare lane 6 with 11). Thus, inefficient activity of the E element correlated with low abundance of the E proteins. To show that the E proteins are indeed required for the activity of the E element, the enhancer activities of the mutants of the E element were determined. Mutants Em-3 and Em-4, whose capacity to bind the E proteins was not significantly affected, displayed enhancer activity, albeit with lower efficiency (21 to 35%; Fig. 3, lanes 3, 5, and 6), whereas mutant Em-5, with impaired binding of the E proteins, exhibited virtually no enhancer activity (lanes 8 and 9).

One of the E proteins was determined to be C/EBP, a liver-restricted transcription factor. However, this element also contains the consensus sequence of the AP-1/*jun* binding site at the region modified in mutant Em-5 (Fig. 2A) (2), raising the possibility that this protein is responsible for the activity of the E element. To demonstrate the binding of AP-1/*jun* to the E element, we used gel retardation competition experiments. Two shifted bands were obtained with nuclear extract of Alexander cells incubated with a ^{32}P-labeled probe that contained the simian virus 40 AP-1/*jun* binding site (AP-1 oligonucleotide) (Fig. 4). A sharp reduction in the shifted bands was observed with the inclusion of 1 ng of unlabeled AP-1 oligonucleotides, but not with E and EP oligonucleotides. However, upon the addition of a larger excess of E oligonucleotide (10 ng), a sharp reduction in the shifted bands was obtained (Fig. 4). We infer that Alexander cells contain *jun* proteins and that the E oligonucleotide binds these proteins with 5- to 10-fold lower affinity than the AP-1 oligonucleotide. Similar data were obtained in reciprocal competition studies in which ^{32}P-labeled E oligonucleotide served as a probe. However, in that case the expected multiple shifted bands (a to e) were obtained, of which only bands b and e were fully subject to competition by the AP-1 oligonucleotide, suggesting that some but not all of the E proteins are *jun* proteins.

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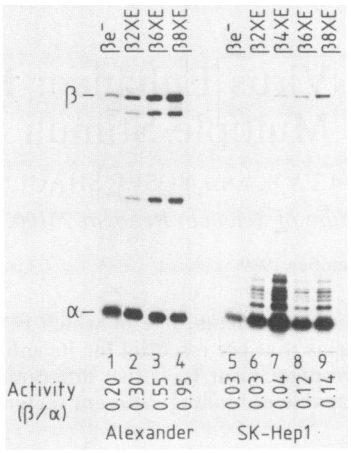

FIG. 1. Activity of synthetic enhancers with progressive increase in E element in different cell lines. Multimeric repeats of synthetic oligonucleotides of the E element containing the sequence shown in Fig. 2A were inserted 2.2 kilobases downstream of the β-globin promoter in the enhancerless vector pβe⁻ (11). Numbers of inserted repeats are shown above the lanes. Cell lines are indicated below the lanes. RNase mapping was performed as described previously (2, 7). α and β, Correctly initiated α- and β-globin transcripts.

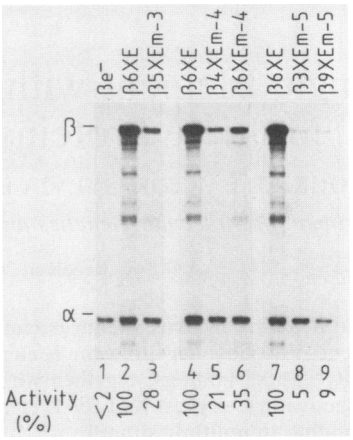

FIG. 3. Activity of E element mutants. Multimers of synthetic oligonucleotides of the E element and its mutant derivatives were cloned at the 5′ end of the β-globin promoter in pβe⁻. Alexander cells were transfected with these plasmids together with plasmid SVHPα2, and after 48 h the RNA was analyzed by RNase A T_1 as described previously (2, 7). Numbers of copies of each oligonucleotide are shown above the lanes. The ratio between β- and α-globin RNA obtained by six copies of E element (β6XE, lanes 2, 4, and 7) was taken as 100% activity.

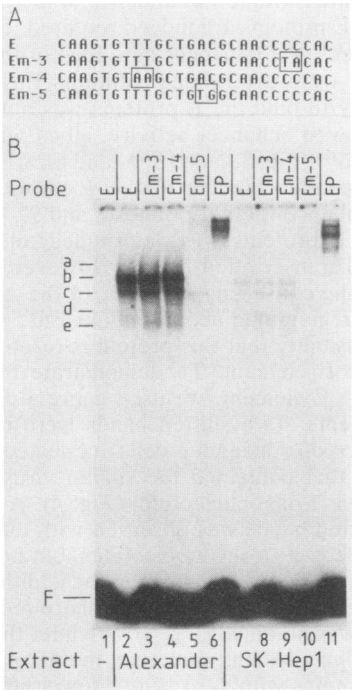

FIG. 2. Gel retardation analysis of the E element and its mutants. (A) Sequences of the synthetic oligonucleotides used. Modified regions are boxed. (B) The complexes formed between the E oligonucleotide ^{32}P-end-labeled probe and mutants with nuclear extracts (5 μg) of either Alexander or SK-Hep1 cells are marked alphabetically. The EP oligonucleotide contains the sequence 5′ CCCCGTTGCTGGGCAACGGCC 3′ as a double-stranded DNA molecule (2). F, Position of free DNA probes.

In order to demonstrate that the binding of *jun* proteins to the E element also has functional significance in living cells, we examined the ability of v-*jun* to induce the enhancer activity of the HBV enhancer (βEN223) and of the E element (β6XE). Cells were cotransfected with a plasmid that directs the synthesis of v-*jun* (SV-v-*jun*, a gift of M. Karin and P. Angel) (1) and a second reporter plasmid. As a positive control we used a reporter plasmid with five copies of oligonucleotide AP-1 (β5XAP-1), and as a negative control we used a reporter plasmid with five copies of EP oligonucleotides (β5XEP). Two different cell lines were analyzed, and v-*jun* activated the E element in both (Fig. 5); however, the effect in SK-Hep1 cells was much more dramatic (18-fold) than it was in Alexander cells (4.5-fold). This difference between the cells was also seen by monitoring the effect of *jun* on an intact HBV enhancer (Fig. 5, lanes 1, 2, 11, and 12). Thus, the effect of *jun* was in full correlation with the relative abundance of the E proteins and confirmed again that in SK-Hep1 cells the poor activity of the E element can

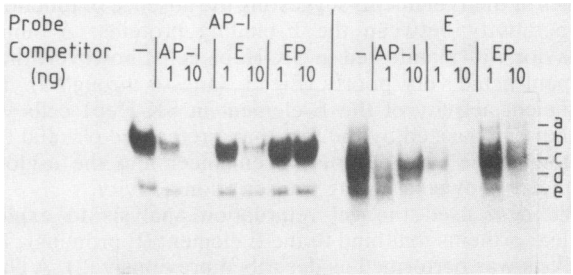

FIG. 4. Binding of *jun* proteins to the E element. The following two oligonucleotides were ^{32}P end labeled and used for a gel retardation assay: AP-1, with the sequence 5′ CGGCTGACTAAT CAAGCA 3′, and E, with the sequence shown in Fig. 2. The probes were incubated with 5 μg of Alexander cell nuclear extract in the absence or presence of homologous and heterologous DNA as competitors. The sequence of EP is given in the legend to Fig. 2. The complexes formed with the E element are labeled alphabetically.

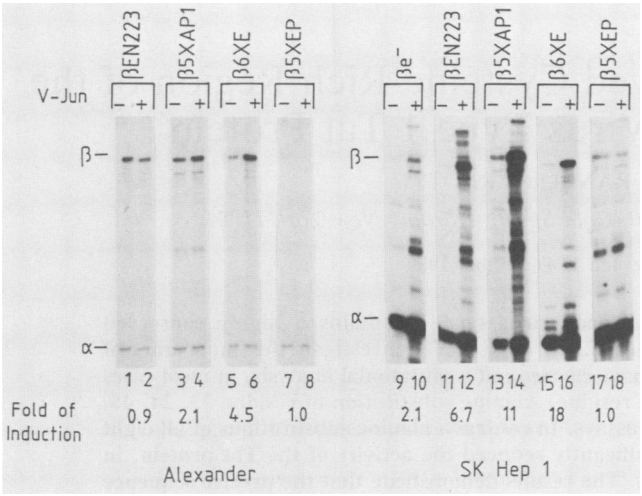

FIG. 5. Induction of HBV enhancer and the E element by v-*jun*. Alexander and SK-Hep1 cells were transfected with βe⁻, βEN223 (containing the HBV enhancer), β6XE (containing six copies of the E element), β5XEP (containing five copies of the HBV enhancer EP element), and β5XAP-1 (containing five copies of the simian virus 40 AP-1 site) either with or without 10 μg of SV-v-jun (a plasmid that directs the synthesis of v-jun; a gift of M. Karin and P. Angel). RNA samples were analyzed by RNase A/TI as described previously (2, 7). Fold induction by v-jun is the ratio between the activity of each plasmid in the presence and absence of SV-v-jun. Activity was calculated by the ratio of β- and α-globin RNAs.

be attributed to the lack of the E proteins. *jun* proteins, in addition to acting as transcription factors, also mediate transcriptional induction in response to phorbol esters (1, 9). It is becoming apparent that *jun* proteins alone or in complex with *fos* (5) are mediators for transmitting the effects of phorbol ester tumor promoters from the cellular membrane to the transcriptional machinery. The binding of *jun* proteins to the E element reported here therefore provides an explanation on the molecular level for the inducibility of this element by phorbol ester tumor promoters (2). However, we have found that the activity of the HBV enhancer is also induced by forskolin, a drug that increases the intracellular level of cyclic AMP (data not shown). The E element, being weakly responsive to forskolin (data not shown), contains a sequence motif highly similar to that of CRE (TGACGCAA). Thus, it is very likely that this element binds the CREB (10) in addition to the *jun* proteins.

An additional protein that binds to the E element is the transcription factor C/EBP (9; R. Dikstein, O. Faktor, and Y. Shaul, submitted for publication). This protein is found mostly but not exclusively in liver cells (3, 4). Although the function of this protein has not been fully revealed, it is assumed that this protein activates promoters and enhancers that bear the corresponding binding site in a tissue-specific manner (4; A. D. Friedman, W. H. Landschulz, and S. L. McKnight, Genes Dev., in press). Thus, the E element binds a tissue-restricted transcription factor in addition to ubiquitous transcription factors, the *jun* proteins, and other related proteins.

Recently, we have found that the X polypeptide of HBV and *tax-1* of human T-cell lymphotropic virus type 1 induce the E element of the HBV enhancer (O. Faktor and Y. Shaul, submitted for publication).

The E element, therefore, is the major HBV enhancer *cis* element via which multiple cellular and viral transcription regulators act. As such, this element plays the role of the enhancer modulator. The capacity of the enhancer to respond to various signals has strong implications for the life cycle of the virus, which is dependent on the proliferative potential of the host. The role of the enhancer modulator (the E element) is to ensure an optimal activity of the enhancer under different physiological conditions. The important role that the E element plays is perhaps the major reason for the strong evolutionary conservation of this element in the genomes of the other members of the hepadnavirus family (2).

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