The EMBO Journal vol.6 no.7 pp.1913-1920, 1987

Multiple nuclear proteins in liver cells are bound to hepatitis B virus enhancer element and its upstream sequences

Yosef Shaul and Rachel Ben-Levy

Department of Virology, The Weizmann Institute of Science, Rehovot 76100, Israel

Communicated by Y.Aloni

The transcriptional enhancer element in the hepatitis B virus (HBV) genome displays tissue-specific activity, suggesting that this element interacts with cellular specific factors. Using a nitrocellulose filter binding assay and DNase I footprinting, we have found that liver cell-specific nuclear proteins are bound to the HBV enhancer element (the E site) and its adjacent sequences. Four DNase I-protected sites were revealed, all contain a sequence motif resembling the sequence of the SV40 enhancer core element. Evidence is provided to show that: (i) these sites are protected by at least three distinct nuclear proteins and (ii) the presence of some of these proteins is dependent on the differentiation stage of the liver cells. Interestingly an octamer sequence found in the E site appears also in the promoter region of several liver-specific genes, which suggests that the E site and its corresponding binding protein(s) determine the tissue-specific expression of the HBV enhancer element.

Key words: hepatitis B virus/DNase I/enhancer element/E site

Introduction

Human hepatitis B virus (HBV) is a member of the hepadna family, that includes viruses which infect liver cells of woodchucks, ground squirrels, tree squirrels and ducks. However, the study of viral life cycle and regulation of viral gene expression is severely hampered, due to the lack of tissue culture systems for efficient viral propagation. Using the chloramphenicol acetyl transferase (CAT) gene system (Gorman *et al.*, 1982) we have previously found and defined an HBV transcriptional enhancer element (Shaul *et al.*, 1985). This enhancer exhibits tissue specific activity and is functional mostly in hepatoma cells (Shaul *et al.*, 1985; Jameel and Siddiqui, 1986; Tur-Kaspa *et al.*, 1986).

The SV40 enhancer consists of multiple elements and the integrated functioning of all of these elements is mediated by cellular *trans*-acting factors (Scholer and Gruss, 1984; Wildeman *et al.*, 1984, 1986; Piette *et al.*, 1985; Sassone-Corsi *et al.*, 1985; Bohnlein and Gruss, 1986; Herr and Clarke, 1986; Zenke *et al.*, 1986). *In vivo* competition studies strongly suggest that HBV enhancer element in conjunction with the promoter of the core gene is also activated by one or more cellular factors (Jameel and Siddiqui, 1986).

Recently several tissue-specific cellular enhancers have been reported. It has been assumed that the action of these *cis* regulatory elements must be mediated by *trans*-acting factors. Indeed, in a few cases either ubiquitous or cell-specific nuclear proteins were found to bind at these sequences (Ohlsson and Edlund, 1986; Sen and Baltimore, 1986; Singh *et al.*, 1986; Weinberger *et al.*, 1986). Ephrussi *et al.* (1985) and Church *et al.* (1985) have obtained footprints of a putative tissue-specific factor in living cells. These nuclear proteins were proposed to be the cellular enhancer *trans*-acting factors, although this possiblility has not yet been experimentally confirmed.

Here we report that liver cell-specific factors are bound at the HBV enhancer element (the E site) and its upstream sequences (the UE sites). Four distinct DNase I-protected regions were defined, and were shown to interact with at least three distinct unrelated factors. We also provide evidence which strongly suggests that some of these factors are differentiation stage specific. The E site is 26 bp long and shows extensive homology with the binding site of EF-C factor at polyomavirus enhancer region (Ostapchuk *et al.*, 1986), with a portion of SV40 72-bp repeat and with the sequence at the promoter region of several liver-specific genes. We propose that the E region is a composite of multiple domains, some of which determine the tissue-specific function of the HBV enhancer element.

Results

Specific binding of nuclear proteins to the HBV enhancer DNA region

Crude nuclear proteins prepared from hepatoma cell nuclei were partially purified for HBV DNA-specific binding proteins as follows: DNA protein binding reaction was performed using large amounts of DNA (500 μg) and nuclear proteins (5 mg), and passed through an RPC-5 column using conditions that retain the DNA protein complex on the column, as described in Materials and methods. After extensive washing of the column, bound proteins were eluted by stepwise increasing the salt concentration in the elution buffer. The obtained fractions were assayed for DNA-specific binding proteins using the nitrocellulose filter binding assay (Riggs *et al.*, 1970). For this purpose, we used an *Xba*I HBV fragment (250-1992), spanning the enhancer element region. This fragment was end labeled and cleaved with *Acc*I (827) enzyme. We found that eluates from the 0.3 and 0.4 M NaCl steps contained protein(s) which specifically bound to the HBV 1.1-kb *Acc*I-*Xba*I (827-1992) DNA fragment (Figure 1). To map further the protein-binding region a DNA-protein binding reaction was performed using the *Nco*I (1375)-digested *Xba*I fragment. Here only the *Xba*I-*Nco*I (249-1375) fragment was retained on nitrocellulose filter. These results suggest that nuclear protein(s) are bound to the *Acc*I-*Nco*I (827-1375) region of HBV genome, the region at which we and others have previously mapped the HBV enhancer element (Shaul *et al.*, 1985; Tognoni *et al.*, 1985). Further digestions of the 1.7-kb *Xba*I fragment with enzymes that cut between the *Acc*I and *Nco*I sites resulted in DNA fragments which indiscriminately bound to the nitrocellulose filter (data not shown). The most logical way to explain these data is to assume the presence of multiple nuclear protein-binding sites at the *Acc*I-*Nco*I region. To test this possibility and to delineate the sequences of the protein-binding sites, we used the DNase I footprinting technique (Galas and Schmitz, 1978).

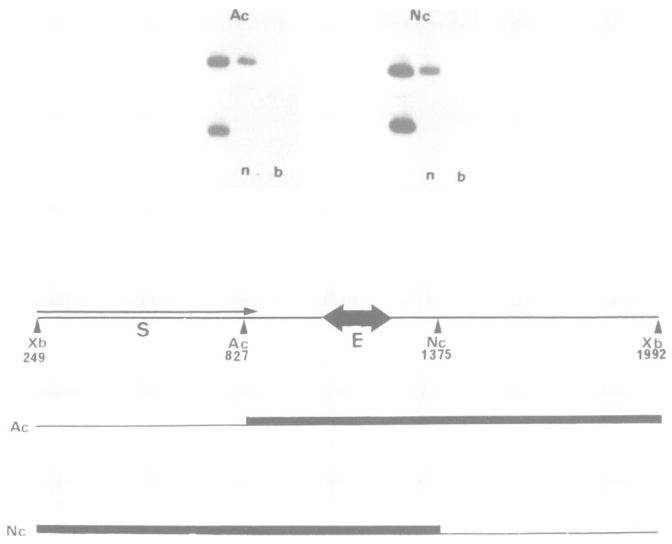

Fig. 1. Detection of nuclear proteins bound specifically to the HBV enhancer region using a nitrocellulose filter binding assay. Crude nuclear extracts were prepared from nuclei of Alexander cells and enriched for HBV enhancer-binding proteins using the RPC-5 column as described in Materials and methods. The *Xba*I (Xb) restriction fragment (249−1992) from HBV subtype adw, containing the enhancer element (E), was labeled at its ends and subjected to second digestion by either *Acc*I (Ac) or *Nco*I (Nc) enzymes. The end-labeled DNA fragments were incubated with the partially purified nuclear extracts prior to filtration through a nitrocellulose filter. The bound fragments were eluted, analysed by agarose gel electrophoresis and exposed to X-ray film as described previously (Shaul *et al.*, 1986). The left lanes show the pattern of DNA before the binding reaction, lanes designated **n** show the binding reactions with partially enriched nuclear extract and **lanes b**, the binding reaction with BSA which served as a negative control. The thick lines represent the fragments bound to the nitrocellulose filter. The thin arrow shows the coding sequence of the S gene.

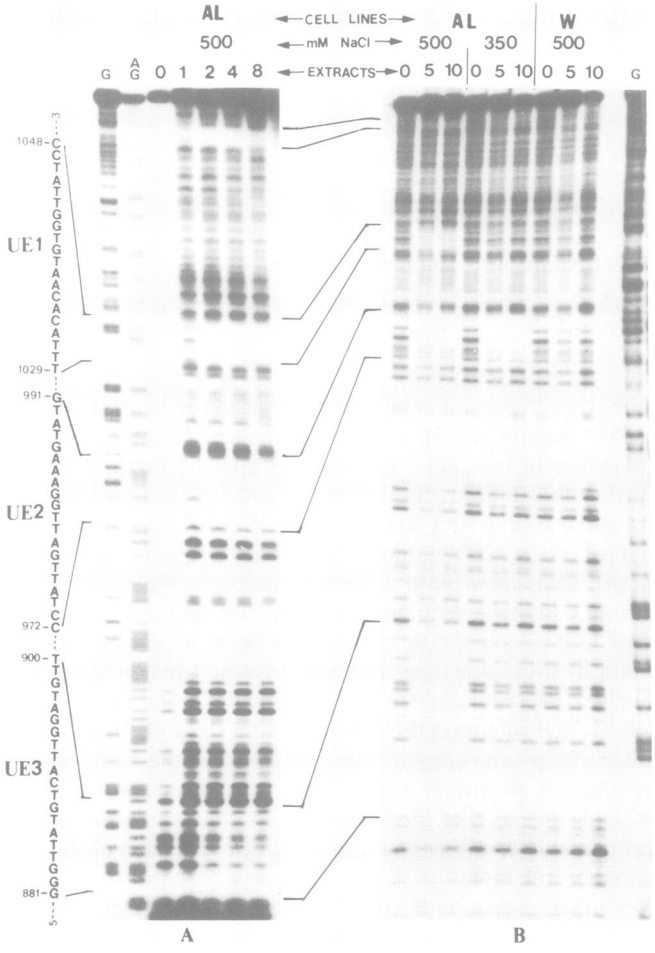

Fig. 2. Footprint analysis of the HBV enhancer region. The *Acc*I (827) *Nco*I (1375) fragment was end labeled at its *Acc*I end and was used in DNase I footprint analysis as detailed in Materials and methods. The extracts were prepared from Alexander cell nuclei (AL) using buffer with 350 mM or 500 mM NaCl and Wish cell nuclei (W) using buffer with 500 mM NaCl. Amounts of extracts that were used are shown in μl on top of each lane. Each microlitre contained ~4.5 μg of proteins. (**A**) A typical footprint pattern, as analysed on a salt gradient sequencing acrylamide urea gel (Biggin *et al.*, 1983) is shown. In this experiment the DNA in the sample without extract (0) was over-digested by the DNase I. (**B**) Two different extracts prepared from Alexander cell nuclei using different salt concentrations, 350 mM and 500 mM NaCl, are compared with that of the extract prepared from Wish cells. The DNase I-protected regions in **A** are aligned to those in **B**. Maxam and Gilbert G and G+A sequencing reactions were run alongside the footprint reaction.

Determination of the sequences of protein-binding sites

A 700-bp restriction DNA fragment which contains the DNA-binding protein sites, as determined by the nitrocelulose filter binding assays, was end labeled at the *Acc*I (827) site, as described in Materials and methods. The DNA fragment was then incubated with various amounts of the crude nuclear extract in the presence of synthetic co-polymer poly(dI)·(dC)−poly(dI)·(dC), which served as non-specific competitor DNA, subjected to partial DNase I digestion, and fractionated alongside sequence markers on salt gradient denaturing polyacrylamide gels (Figure 2).

In accordance with the result of the nitrocellulose filter binding assays, several regions of the HBV enhancer and adjacent sequences were protected from DNase I digestion as the amount of the nuclear extract was increased. The sequences of the protected regions of three of these sites are shown next to the autoradiograms (Figure 2). The site designated UE3 is positioned at nucleotides 881 to 900, ~180 bases upstream from the enhancer element, previously mapped at region 1080−1234 (Shaul *et al.*, 1985; Tognoni *et al.*, 1985). The second DNase I-protected region, designated UE2, is positioned at nucleotides 972−991, ~72 bp downstream from UE3 and 89 bp upstream from the enhancer element. UE2 is the only DNase I-protected site that was detected with nuclear extract prepared at 350 mM NaCl (Figure 2B). This observation suggests that the UE2 factor is different from the others. This will be confirmed using different expeirmental approaches (see below). The third DNase I-protected region, designated UE1, is located ~38 bp downstream from UE2 at nucleotides 1029−1048. An additional DNase I-protected region is seen at the top of the autoradiogram, where the resolution of the sequencing gel was not good enough to determine the DNA sequence. Therefore in order to delineate the sequence of this protected region, we used a second probe, which was end labeled next to the *Stu*I (1115) site. A distinct region was protected from DNase I digestion as increasing amounts of nuclear extract prepared from Alexander cells were added (Figure 3). This region is 24 bp, from nucleotide 1181 to 1204 and is designated E. The same region was also protected when a second fragment labeled at the opposite end was used for this assay (data not shown). The E region maps directly within the HBV enhancer element, between its two GC-rich domains, where a sequence resembling the SV40 enhancer element core sequence was previously noted (Shaul *et al.*, 1985). All these four DNase-protected regions were not detected when proteinase

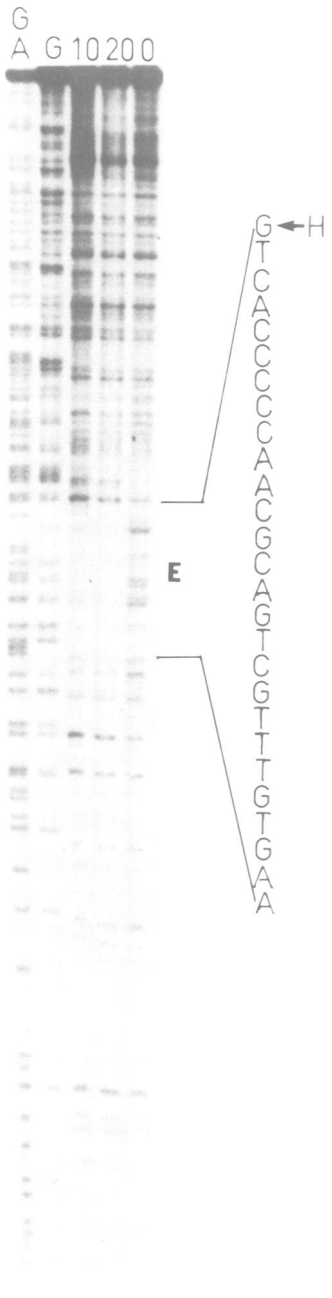

Fig. 3. Footprint analysis of the HBV enhancer. A *Stu*I−*Hin*cII (1115−1685) fragment of HBV DNA subtype adw was cloned in the multiple cloning site of pSP6 and subsequently was digested with *Eco*RI, which cuts in the multiple cloning site of the plasmid next to the *Stu*I site, and end-labeled. This DNA was incubated with increasing amounts of Alexander cell nuclear extracts prior to exposure to partial digestion with DNase I. The sequence of the E-protected site is shown, and the DNase I-hyperdigested site (H) is marked by an arrow.

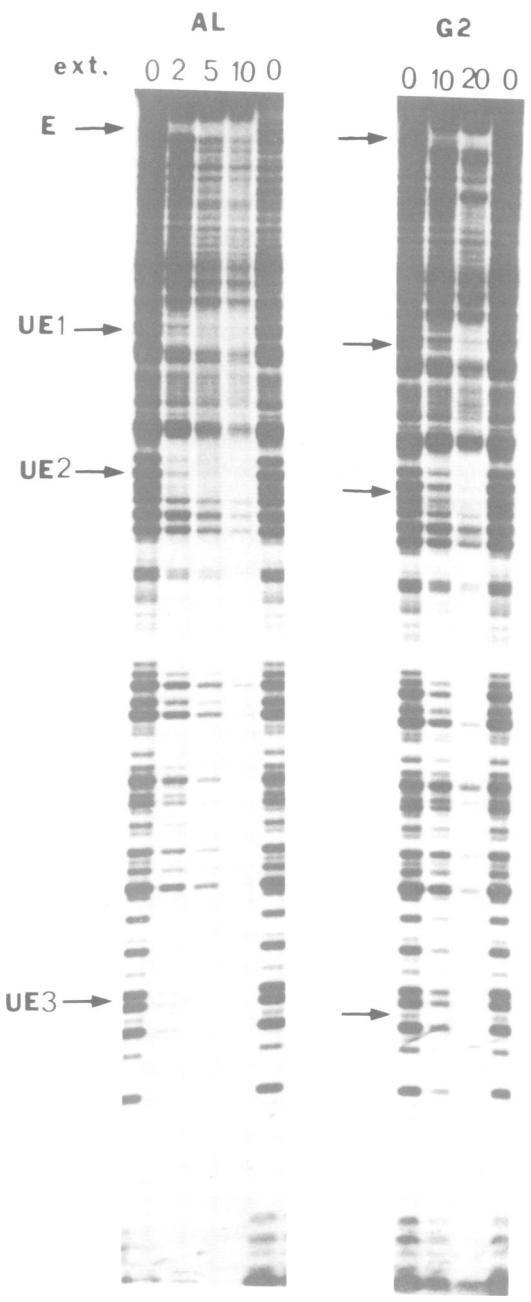

Fig. 4. Footprint analysis with nuclear extract prepared from HepG2 cell line. The end-labeled DNA fragment described in Figure 2 was incubated with increasing amounts of nuclear extracts prepared from Alexander cells (AL) and HepG2 cells (G2). The nuclear extracts were prepared using buffer containing 500 mM NaCl. The amounts of extracts that were used are shown in μl. Each microlitre of Alexander cell nuclear extract contains ~5 μg protein while that of HepG2 cells ~2 μg. The protected regions are shown by arrows.

K-treated extracts were used (data not shown), therefore we will refer to these regions as protein-binding sites.

We subsequently addressed the question whether the appearance of these HBV DNA-binding nuclear proteins are tissue specific as we had observed earlier in terms of HBV enhancer activity. For this purpose we used nuclear proteins prepared from Wish cells, a human amnion cell line, for comparison with Alexander cells. When increasing amounts of Wish cell extract were incubated with end-labeled HBV DNA fragment, no DNase I-protected region was obtained (Figure 2B). This suggests that nuclear protein(s) bound to HBV DNA at the enhancer region are cell type specific.

The HBV DNA binding proteins are of cellular origin

Alexander cells, used in these studies, are known to contain integrated forms of HBV DNA and to express constitutively HBsAg (Macnab *et al.*, 1976). Therefore, the nuclear proteins that bind to the HBV enhancer region may be either of cellular or viral origin (coded or induced by virus). To distinguish between these two possibilities we analysed the HepG2 cell line (Knowles *et al.*, 1980). HepG2 is a differentiated human hepatoma cell line,

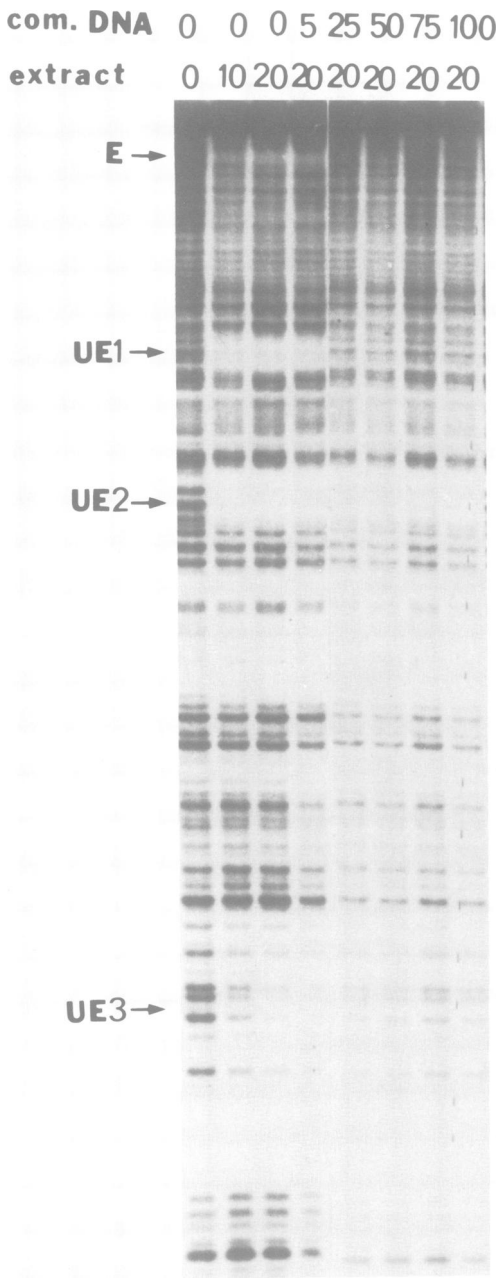

Fig. 5. Footprint competition analysis of E site. The footprinting experiment was performed as described in Materials and methods and the legend to Figure 2. About 3 ng of end-labeled *Acc*I–*Nco*I fragment was mixed with 20 μg of Alexander cell nuclear extracts and increasing amounts of the competitor DNA indicated at the top of the figure in ng. For the competitor DNA we used a gel-purified *Stu*I (1115) *Bam*HI (1403) DNA fragment containing only the E-binding site.

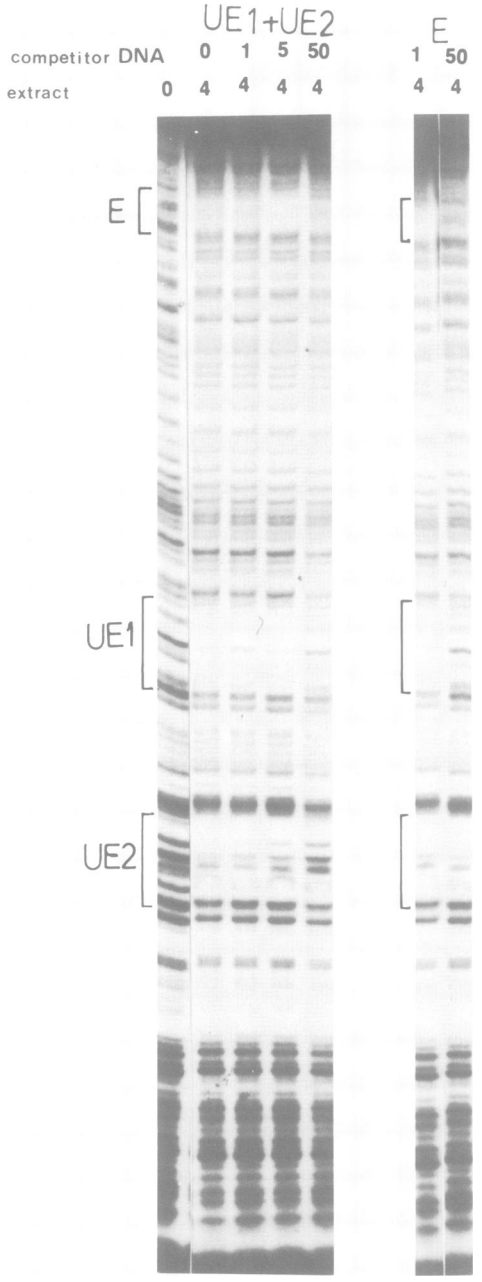

Fig. 6. Footprint competition analysis of UE1 and UE2 sites. The experiment was performed as described in the legend to Figure 2 except that 4 μl of extracts were used and the competitor DNA is a *Stu*I fragment (980–1115) containing the UE1 and UE2 sites. In this set of experiments the UE3 site was not detected. In parallel, at the right side of the figure we show the results of the experiment in which the competitor DNA described in the legend to Figure 5 was used.

but unlike Alexander cells, this cell line does not contain integrated or extrachromosomal forms of HBV DNA. Nuclear extracts were prepared from Alexander and HepG2 cells and incubated with an *Acc*I (827) end-labeled HBV DNA fragment and subjected to partial digestion with DNase I (Figure 4). The DNase I footprint patterns of these two cell lines are identical, suggesting that these proteins are of cellular origin. To estimate approximately the amount of these proteins in both cell lines, we compared the intensity of the bands at the protected area when equal amounts of nuclear extracts were used. Assuming that the binding affinities of these proteins are the same in both cell types, there is no substantial difference in their quantities.

Competition footprint analysis of the HBV enhancer region

An experimental approach to determine the relationshp between the footprint-protected sites at the enhancer region is the competition footprint technique. Footprint analysis, using an *Acc*I end-labeled probe, was carried out in the presence of unlabeled competitor HBV DNA containing only the E region. Addition of this unlabeled DNA severely inhibited not only formation of the E footprint, as expected, but also the formation of the UE1, whereas the other two adjacent binding regions UE2 and UE3 were not affected (Figure 5). Next we used as a competitor DNA a fragment that contains only the UE1 and UE2 sites (Figure 6). In

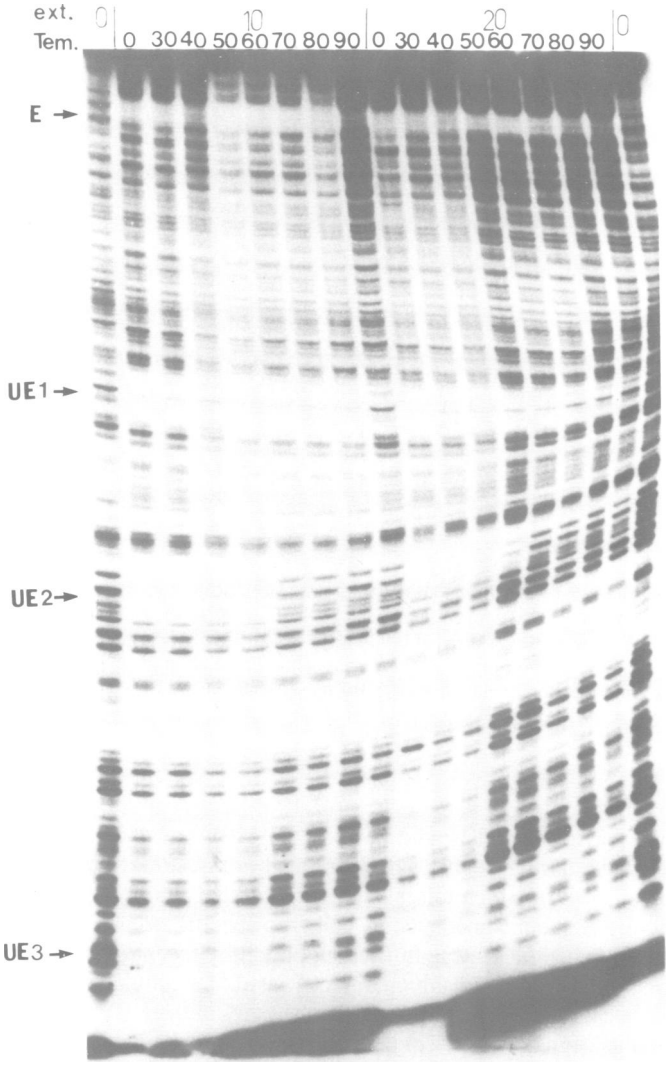

Fig. 7. Determination of temperature sensitivity of the HBV DNA-binding cellular factors. 0, 10 and 20 μl of nuclear extracts prepared from Alexander cells were used for each assay. Each sample was heat treated at the indicated temperature for 10 min, immediately cooled and spun in an Eppendorff centrifuge for 2 min before being used for the DNase I-footprinting assays. Salt gradient sequencing gel was used in this experiment.

this case formation of the UE1 and UE2 footprints were inhibited, whereas the E footprint was almost unaffected. Under similar conditions, when unlabeled DNA containing the E site was used, the E and UE1 footprints were severely inhibited. These results demonstrate that region E efficiently competes for the factor(s) bound to UE1 site, but the UE1 site does not compete for the factors which bind to the E site. One possible explanation is to assume that each site binds a different factor and that the two factors bind co-operatively. Thus, the E site should be occupied in order to allow the binding of the UE1 in a manner that was previously proposed in other systems (Ptashne, 1986). It is also clear from competition footprint analysis that the protein bound to UE2 is different from those binding to E and UE1, since no cross competition was observed between these sites. This finding is consistent with our initial observation that low salt extract contains only the UE2 factor (Figure 2). The data, however, are not conclusive for the UE3 site. The major problem with UE3 is that this site was not always detectable. It is possible that a protein bound to UE3 site is not stable and therefore its activity varies from preparation to preparation.

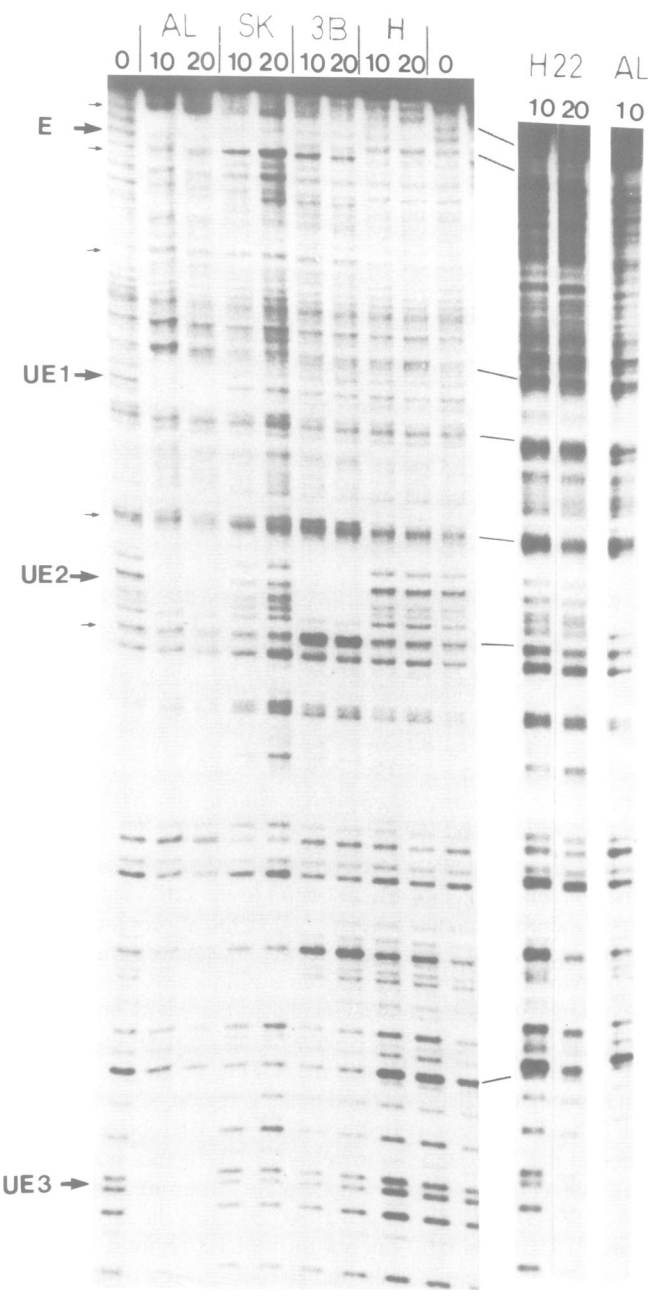

Fig. 8. Cell type specificity of HBV footprint patterns. Nuclear extracts were prepared from Alexander cells (AL), SK-Hep1 cells (SK), Hep3B cells (3B), HeLa cells (H) and HA22T/VGH cells (H22) using buffer with 500 mM NaCl. The *Acc*I−*Nco*I end-labeled fragment was incubated with 10 and 20 μl of nuclear extract before being subjected to partial DNase I digestion. The four protected sites are marked by big arrows and the DNAse I-hyperdigested site by small arrows.

Heat sensitivity of the HBV enhancer cellular factors

One additional way to define the composition of the factors bound to HBV enhancer region is to determine their heat sensitivity. Extracts were subjected to heat treament for 10 min prior to incubation with an end-labeled probe for a DNase I footprinting experiment (Figure 7). The E, UE1 and UE3 factors are relatively heat stable. Only at temperatures up to 80°C was their binding activity inactivated. On the other hand, the factor(s) bound to UE2 is heat sensitive and inactivated at ~60°C. In accordance with the footprint competition experiments, these data again suggest that the UE2 protein is distinct from the others.

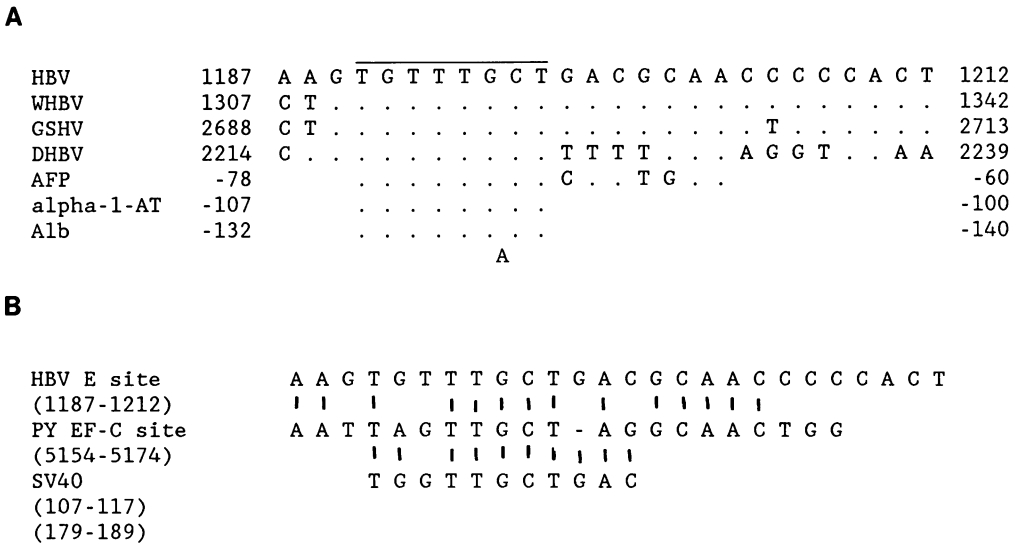

Fig. 9. Sequence homologies to the HBV E site. (**A**) The sequence of the HBV E site is compared with the known viral sequences of the following members of the hepadna family: woodchuck hepatitis B virus (WHBV), ground squirrel hepatitis B virus (GHBV) and duck hepatitis B virus (DHBV), and the promoters of the following liver-specific genes: α-fetoprotein (AFP; Godbout *et al.*, 1986), α_1-antitrypsin (α_1-AT; Ciliberto *et al.*, 1985) and human albumin (Alb). The homologous bases are indicated by a dot. The consensus sequence is marked by a line on top of the sequence of the E site. The numbering refers to the position of these sequences in the viral genomes or to their distances from the transcriptional initiation sites. (**B**) The sequence of the HBV E site is compared with that of the polyoma virus EF-C site (Ostapchuck *et al.*, 1986) and to that of the SV40 72-bp repeat.

Some of the cellular factors which bind to the HBV DNA are differentiation-stage specific

We have shown that the differentiated, Alexander and HepG2 cells contain the factors which bound to the HBV enhancer element. One obvious question is whether the appearance of these factors is differentiation-stage specific. To answer this question, we prepared nuclear extracts from a number of hepatoma cell lines and from HeLa cells and incubated them with an *Acc*I end-labeled probe for a DNAse I footprinting assay (Figure 8). SK-Hep1 the undifferentiated hepatoma cell line (Chang *et al.*, 1983), contains only the factor(s) bound to the E region, suggesting again that E binding factor is distinct from the UE1 binding factor, although the E sequence competes for the factors bound to the UE1 site (Figure 7). The moderately differentiated cell line (HA22T/VGH) (Chang *et al.*, 1983) contains the factors bound to the E, UE1 and UE3 but not to the UE2 regions. Similar to Alexander and HepG2 cells, the Hep3B cell line – highly differentiated liver cells (Knowles *et al.*, 1980) – contains substantial amounts of UE2 but does not contain the UE1 and UE3 factors. These data clearly demonstrate that: (i) UE2 is expressed only in highly differentiated liver cells, (ii) the UE2 factor is distinct from E, UE1 and UE3, and (iii) the E factor is distinct from UE1 and UE3 and expressed in all the tested liver cell lines regardless of their stage of differentiation.

In addition to appearance of gaps at the protein-binding sites after DNase treatment, enhanced DNase I cleavage sites next to the binding region are also accompanied by DNA–protein interactions. Several liver cell-specific DNAse I hypersensitive sites are clearly seen in Figure 8. Of most interest is the site at the 5′ end of the E region. This DNase hypersensitive site does not appear when nuclear extract from HeLa cells was used, although apparently the E region was partially protected. Thus the HeLa cell factor that bound to E site might be related but not identical to that found in liver cells.

Discussion

We have previously shown that the HBV enhancer element displays tissue-specific activity (Shaul *et al.*, 1985; Tur-Kaspa *et al.*, 1986), which raises the possibility that activity of this *cis*-acting element is mediated by cell type-specific *trans*-acting factor(s). This notion was further supported by recent *in vivo* competition experiments which demonstrated that cellular factors in liver cells are required for efficient activity of the HBV enhancer and its cognate promoter (Jameel and Siddiqui, 1986). In attempts to search for a cellular factor (or factors) which interacts directly with the HBV enhancer element, we prepared nuclear extracts and performed nitrocellulose filter binding assays (Riggs *et al.*, 1970) and DNAse I footprinting experiments (Galas and Schmitz, 1978). Four clearly protected sites were detected in the region of the HBV enhancer element (the E site) and at its adjacent sequence (the UE1-3 sites). We suggest that at least three distinct factors are involved in the apparent protection results. This suggestion is based on: (i) the salt concentration used to leach the factors from the nuclei; (ii) competition footprint analysis; (iii) differences in the thermostability of the factors; and (iv) their cell-specific appearance. One factor is bound to the E site, the second to the UE1 and probably to the UE3 sites and the third to the UE2 site. E factor was found in all tested hepatoma cells, regardless of their stage of differentiation. Also, a related but possibly not identical nuclear protein which interacts with this site was found in the nuclei of HeLa cells. UE2 factor, on the other hand, was found only in differentiated (Alexander, HepG2 and Hep3B) cells, but not in moderately or undifferentiated hepatoma cells. The factor which binds to UE1 (and possibly to UE3 sites) is not detectable in poorly differentiated cells but is found in the moderately and highly differentiated hepatoma cells with the exception of Hep3B cells. The absence of this factor in Hep3B-differentiated cells may imply that its expression is not exclusively linked to the differentiation stage.

In absence of an *in vitro* system to assess the activity of the enhancer element propagation, it is not a simple task to determine the role and the requirements of each of these factors in enhancer activity. We have compared the sequence of E sites among the members of hepadna virus and have found that it is highly conserved with the exception of DHBV (Figure 9). This evolutionary conservation of the E site and most likely of the E factor suggests that this protein plays an important role in liver cells. Indeed we have recently found that the liver of normal rabbits contains a protein which can bind to the E site (I.Berger and Y.Shaul, unpublished observation). This assumption is further supported by sequence homology found between the E site and the promoter of α-fetoprotein (AFP) gene (Figure 9). The AFP gene is active mainly in fetal liver and in liver cell lines. Extensive analysis of the promoter and the enhancer elements of this gene revealed that this tissue-specific function of AFP gene must reside next to the promoter sequences, in a region between −85 and −33 (Godbout *et al.*, 1986). Most interestingly, 12 out of 15 bp of this region are homologous to that of the E site. Furthermore, an octamer sequence of the E site (TGTTTGCT) is found next to the promoter region of some other liver-specific genes (Figure 9). Thus we would like to propose that this octamer sequence plays an essential role in determination of liver-specific function of these genes and perhaps of the HBV enhancer element.

Recently, a ubiquitous cellular factor, named EF-C, was found to interact with the polyomavirus enhancer region (Ostapchuk *et al.*, 1986). Surprisingly the sequence of the binding site of this factor is highly homologous to that of the E site (Figure 9). This sequence homology strongly suggests that EF-C or a similar factor binds to the E site and may in fact be the factor which we have found to bind to the E site using HeLa cell extract (Figure 8). Sequence homology was also found with SV40 enhancer element, 10 out of the first 11 bp of the 72-bp repeat of SV40 enhancer element are homologous to the E site. It appears, therefore, that the E site is a composite of multiple binding domains some of which may determine the tissue-specific function of the HBV enhancer element.

The possibility that short stretches of DNA sequence serve as a common binding site for both ubiquitous and tissue-specific nuclear proteins is reminiscent of the SV40 enhancer element (Davidson *et al.*, 1986) and the octameric sequence of the immunoglobulin promotors (Landolfi *et al.*, 1986; Staudt *et al.*, 1986), and it seems to be of biological significance.

The SV40 enhancer core sequence GTGGA/TA/TA/TG was suggested to play a key role in activity of the enhancer (Weiher *et al.*, 1983; Herr and Clarke, 1986; Zenke *et al.*, 1986). Both the A and C domains of the SV40 enhancer element described by Herr and Clarke (1986) share homology with the core sequence and the B domain contains six out of eight nucleotides. This core sequence was also found in a number of enhancer elements of cellular genes, some of which display tissue-specific activity, and cellular factors which interact with the core element were recently described (Ohlsson and Edlund, 1986; Wildeman *et al.*, 1986). The finding that this core element sequence in the insulin gene enhancer is recognized by tissue-specific cellular factors suggests that there is no general core element-binding factor. This suggestion is further supported by our findings which clearly demonstrate that in a given cell type there are three distinct factors which interact with sequences resembling the core element: site E, UE1 and UE2 each contains seven out of eight nucleotides and site UE3 contains six out of eight. It might appear that these factors belong to a family of proteins which have some similarity in their DNA-binding domain. In this regard, it is interesting to mention the homeotic genes. This family of genes shares a highly conserved protein domain, known as the homeo box (Gehring, 1985). Recently it was shown that this domain has DNA-binding activity (Desplan *et al.*, 1985). The possibility that the products of these genes activate tissue and differentiation stage-specific enhancers is intriguing and requires further study.

Materials and methods

Cell growth

The following cell lines were used, PLC/PRF/5 (Alexander cells) (Macnab *et al.*, 1976), HepG2 and Hep3B (Knowles *et al.*, 1980), SK-Hep1 and HA22T/VGH (Chang *et al.*, 1983), HeLa cells and Wish cells (ATCC CCL 25). Cells were cultured in Dulbecco's modified Eagle's minimal essential medium (GIBCO) containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Preparation of nuclear extracts

Nuclear extracts were prepared using the method described by Siebenlist *et al.* (1984), except that either 350 or 500 mM NaCl was included in buffer B and the step of $(NH_4)_2SO_4$ precipitation was omitted (Shaul *et al.*, 1986). The protein concentration was typically 3−6 μg/μl.

RPC-5 column purification of nuclear extracts

About 500 μg of PCP10 plasmid containing a dimer of HBV DNA (Pourcel *et al.*, 1982) was mixed with 0.5−1 mg of nuclear extracts prepared from Alexander cells in 1 ml of binding buffer containing 150 mM NaCl (Shaul *et al.*, 1986) without DNA carrier, at 0°C for 30 min. The reaction mixture was applied onto a 0.5 g RPC-5 column that was pre-adjusted to the binding buffer. The column was washed with 5 ml of binding buffer followed by elution with binding buffer with stepwise increase of the salt concentration up to 0.5 M. Aliquots of the obtained fractions were assayed for HBV-specific DNA-binding proteins using end-labeled HBV DNA fragments and the nitrocellulose filter binding assay (Riggs *et al.*, 1970), HBV-specific binding proteins were usually obtained in fractions with 350−400 mM NaCl. Although these fractions gave clear results with nitrocellulose filter binding assays, they were not good enough for the footprinting reactions.

DNase I footprinting

The preparation of end-labeled DNA fragments and the conditions for performing footprinting reactions using crude nuclear extracts and poly(dI)·(dC)−poly(dI)·(dC) as DNA carrier are described elsewhere (Shaul *et al.*, 1986). In some cases salt gradient sequencing acrylamide urea gels were used for analyses of the footprinting reactions (Biggin *et al.*, 1983).

Acknowledgements

We thank Drs O.Laub, D.N.Standring and O.Resnekov for critical reading of the manuscript and Dr C.Chang for the HA22T/VGH cell line. This work was supported by grants from the Leukemia Research Foundation, Chicago, Israel Cancer Association and the Israel Research Foundation.

References

Biggin,M.B., Gibson,T.J. and Hong,G.F. (1983) *Proc. Natl. Acad. Sci. USA*, **81**, 1991−1995.
Bohnlein,E. and Gruss,P. (1986) *Mol. Cell Biol.*, **6**, 1401−1411.
Ciliberto,G., Dente,L. and Cortese,R. (1985) *Cell*, **41**, 531−540.
Chang,C., Lin,Y., O-Lec,T.W., Chou,C.K., Lee,T.S., Liu,T., Peng,F.K., Chen,T.Y. and Hu,C.P. (1983) *Mol. Cell. Biol.*, **3**, 1133−1137.
Church,G.M., Ephrussi,A., Gilbert,W. and Tonegawa,S. (1985) *Nature*, **313**, 798−801.
Desplan,C., Thais,J. and O'Farrell,P.H. (1985) *Nature*, **318**, 630−635.
Davidson,I., Fromental,C., Augereau,P., Wildeman,A., Zenke,M. and Chambon,P. (1986) *Nature*, **323**, 544−548.
Ephrussi,A., Church,G.M., Tonegawa,S.W. and Gilbert,W. (1985) *Science*, **227**, 134−140.
Galas,D. and Schmitz,A. (1978) *Nucleic Acids Res.*, **5**, 3157−3170.
Gehring,W.J. (1985) *Cell*, **40**, 3−5.
Godbout,R., Ingram,R. and Tilghman,S.M. (1980) *Mol. Cell. Biol.*, **6**, 477−487.

Gorman,C.M., Moffat,L.F. and Howard,B.H. (1982) *Mol. Cell Biol.*, **2**, 1044–1051.
Herr,W. and Clarke,J. (1986) *Cell*, **45**, 461–470.
Jameel,S. and Siddiqui,A. (1986) *Mol. Cell Biol.*, **6**, 710–715.
Knowles,B., Howe,C.C. and Aden,D.P. (1980) *Science*, **209**, 497–501.
Landolfi,N.F., Capra,J.D. and Tucker,P.W. (1986) *Nature*, **323**, 548–551.
Macnab,G.M., Alexander,J.J., LeCatsa,E.M., Bey,D. and Urbanowitz,J.M. (1976) *Br. J. Cancer*, **34**, 509–515.
Ohlsson,H. and Edlund,T. (1986) *Cell*, **45**, 35–44.
Ostapchuk,P., Diffley,J.F.X., Bruher,J.T., Stillman,B., Levine,A.J. and Hearing,P. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 8550–8554.
Piette,J., Kryszyke,M.H. and Yaniv,M. (1985) *EMBO J.*, **4**, 2675–2685.
Pourcel,C., Louise,A., Gervais,M., Chenciner,N., Dubois,M.F. and Tiollais,P. (1982) *J. Virol.*, **42**, 100–105.
Ptashne,M. (1986) *Nature*, **322**, 697–701.
Riggs,A.D,. Bourgeois,S. and Cohn,M. (1970) *J. Mol. Biol.*, **53**, 401–417.
Sassone-Corsi,P., Wildeman,A. and Chambon,P. (1985) *Nature*, **313**, 458–463.
Scholer,H.R. and Gruss,P. (1984) *Cell*, **36**, 403–410.
Sen,R. and Baltimore,D. (1986) *Cell*, **46**, 705–716.
Shaul,Y., Rutter,W.J. and Laub,O. (1985) *EMBO J.*, **4**, 427–430.
Shaul,Y., Ben-Levy,R. and De-Medina,T. (1986) *EMBO J.*, **5**, 1967–1971.
Siebenlist,U., Hennighausen,L., Battey,J. and Leder,P. (1984) *Cell*, **37**, 381–391.
Singh,H., Sen,R., Baltimore,D. and Sharp,P.A. (1986) *Nature*, **319**, 154–158.
Staudt,L.M., Singh,H., Sen,R., Wirth,T., Sharp,P.A. and Baltimore,D. (1986) *Nature*, **323**, 640–643.
Tognoni,A., Cattaneo,R., Serfling,E. and Schaffner,W. (1985) *Nucleic Acids Res.*, **13**, 7457–7464.
Tur-Kaspa,R., Burk,R.D., Shaul,Y. and Shafritz,D.A. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 1627–1631.
Weiher,H. Konig,M. and Gruss,P. (1983) *Science*, **213**, 626–631.
Weinberger,J., Baltimore,D. and Sharp,P.A. (1986) *Nature*, **322**, 846–848.
Wildeman,A.G., Sassone-Corsi,P., Grundstrom,T., Zenke,M. and Chambon,P. (1984) *EMBO J.*, **3**, 3129–3133.
Wildeman,A.G., Zenke,M., Schatz,C., Wintzerith,M., Grundstrom,T., Matthes,H., Takahashi,K. and Chambon,P. (1986) *Mol. Cell Biol.*, **6**, 2098–2105.
Zenke,M., Grundstrom,T., Matthes,H., Wintzerith,M., Schatz,C., Wildeman,A. and Chambon,P. (1986) *EMBO J.*, **5**, 387–397.

Received on February 10, 1987; revised on April 13, 1987