Differences in the target specificity of the transactivating factors MHBs¹ and HBx of hepatitis B virus

Edith Mühlbauer and Werner Koch*

Max-Planck-Institut für Biochemie, Department of Virus Research, Am Klopferspitz 18a, D-82152 Martinsried, Germany

Transient transfections of tissue culture cells with plasmids encoding the transactivating factors MHBs¹ and HBx of hepatitis B virus result in transcriptional stimulation of multiple target genes. Our experiments show that the NF-κB-binding enhancer element of simian virus 40 (SV40) and the AP-1-binding enhancer element of the human metallothionein II A gene mediate the transactivating function of MHBs¹ and HBx. In contrast, the elements GT(IIC + I) and Sph(II + I) of the SV40 enhancer, that, as a common feature, require binding of transcription factor TEF-1 for activity, efficiently mediate transactivation only by HBx but not by MHBs¹. This finding suggests that at least one regulatory pathway exists that can only be activated by HBx but not by MHBs¹.

Experiments with tissue culture cells revealed that carboxy-terminally truncated middle surface antigen (MHBs¹) and HBx of hepatitis B virus (HBV) are able to act as transcriptional transactivators (Twu & Schloemer, 1987; Wollersheim et al., 1988; Zahm et al., 1988; Caselmann et al., 1990; Kekulé et al., 1990). The transactivating function of MHBs¹ and HBx is directed to many known viral and cellular enhancer and promoter elements. Synopsis of epidemiological and molecular studies led to the assumption that, possibly based on their transactivating property, MHBs¹ and HBx contribute to the onset or development of hepatocellular carcinoma in individuals who, as a consequence of previous infection with HBV, carry chromosomally integrated viral DNA (for reviews see Tiollais et al., 1985; Ganem & Varmus, 1987; Beasley, 1988; Koshy & Meyer, 1992).

The molecular mechanism that underlies the transactivating function of MHBs¹ and HBx is not known. Recent observations suggested that the generation of reactive oxygen intermediates is required for the activation of nuclear factor- (NF) κB by MHBs¹ and HBx, and that protein kinase C is involved in the stimulation of activator protein-1 (AP-1) by HBx (Meyer et al., 1992; Kekulé et al., 1993). It has not been directly examined whether MHBs¹ and HBx have differences in their target specificities. In theory, the existence of at least one enhancer element that can be stimulated only by one but not by the other of the HBV-derived transactivators would indicate the existence of, at least in part, alternate cellular signal transducing pathways for MHBs¹ and HBx.

To address this issue, we directly compared the ability of MHBs¹ and HBx to perform their transactivating function, using selected, transcription factor-binding sequence elements present in the enhancer of simian virus 40 (SV40) and in the enhancer of the human metallothionein II A (hMT II A) gene. Chang liver cells (Chang, 1954) were cotransfected with transactivator plasmid or non-transactivating control plasmid and one of several reporter plasmids. Transactivator plasmids were pMHBs¹, encoding MHBs¹ and pHBV824, encoding HBx. Control plasmids were pMHBs¹, encoding full-length MHBs¹ and pHBV824fs, encoding, in theory, a short amino-terminal fragment of HBx and non-transactivating control plasmid and one of several reporter plasmids. Transactivator plasmids were pMHBs¹, encoding MHBs¹ and pHBV824, encoding HBx. Control plasmids were pMHBs¹, encoding full-length MHBs¹, and pHBV824fs, encoding, in theory, a short amino-terminal fragment of HBx and amino-terminally truncated fragments of HBx initiating at methionine residues 79 and 103 (Fig. 1; see also Luber et al., 1991; Kwee et al., 1992; Lauer et al., 1992). However, as shown by mutation and deletion analyses, these HBx fragments are unable to activate RNA polymerase II-dependent regulatory elements (Ritter et al., 1991; Arii et al., 1992; Kwee et al., 1992; Kim et al., 1993; Renner et al., 1995). The reporter plasmids, containing the rabbit β-globin promoter and the β-globin gene as a reporter gene, are based on pG1 (Fig. 2; see also Fromental et al., 1988; Kanno et al., 1989; Luber et al., 1991). The pG1
Fig. 1. Schematic representation of the transactivator plasmids pSVMHBs and pHBV824 and the non-transactivating control plasmids pSVMHBs and pHBV824fs. Construction of the plasmids is described in Luber et al. (1991) and Lauer et al. (1992). Plasmid pSVMHBs was originally named as pSVMHBs (Lauer et al., 1992). SV40 indicates the position of the SV40 enhancer and early promoter. Key: p(A), polyadenylation signal; E I, enhancer I; XP, X gene promoter.

Fig. 2(a). For legend see opposite.

recombinants carry either the entire SV40 enhancer, as in pG1B, or specific enhancer elements arranged in tandem repeats in front of the β-globin promoter and β-globin gene. As shown previously, these enhancer elements function as specific binding sites for the transcription factors NF-κB, AP-1, AP-2, transcriptional enhancer
Fig. 2. (a) Structure of the SV40 enhancer and early promoter region and the reporter plasmids pG1 and pG1B. The enhancerless plasmid pG1 contains the rabbit β-globin gene (from −109 to +1650), pBR322 vector sequences (from 2066 to 4363) and a polylinker (EcoRI–SmaI–XbaI–SstI–XhoI–BamHI) upstream of the β-globin gene (Fromental et al., 1988). Plasmid pG1B contains the PvuII–BamHI fragment (SV40 enhancer sequences) of pA0 inserted into the XhoI site of pG1 (Zenke et al., 1986; Fromental et al., 1988). The striated boxes in pG1B indicate the locations of functional sequence elements that were defined on the basis of previous in vitro protein binding studies and in vivo mutagenesis studies (Angel et al., 1987; Lee et al., 1987; Davidson et al., 1988; Fromental et al., 1988; Kanno et al., 1989; Macchi et al., 1989). The transcription factors binding to these functional elements are shown in brackets. The locations of the SV40 enhancer elements outlined in (b) are shown below pG1B. Key: LES, ‘late early start’; EES, ‘early early start’. (b) Structure of the TC-II, TC(II + I), P (SVP), GT(IIC + I), and Sph(II + I)+ Oct elements of the SV40 enhancer and the AP-1-binding element (MTP) of the enhancer of the human metallothionein IIA gene. The nucleotide sequences of the double-stranded wild-type templates are shown along with the locations of the point mutations of the mutant templates. The SV40 coordinates (BBB numbering system; Tooze, 1982) are shown above the sequence and the name of each recombinant is to the left. The nucleotides in boxes represent the functional sequence elements. The sequences shown constitute the units that were tandemly repeated four times. The repeated units were inserted between the XhoI and SrfI sites of the polylinker of pG1 in an orientation opposite to that existing in the SV40 enhancer with the exception of TC-I(II)M13 which was inserted in the same orientation. Key: OBP, octamer-binding protein.

factor- (TEF) 1, TEF-2 and octamer-binding proteins (Angel et al., 1987; Lee et al., 1987; Mitchell et al., 1987; Rosales et al., 1987; Davidson et al., 1988; Macchi et al., 1989). Data from protein binding studies and transfection experiments has shown that the interaction between the enhancer elements and their respective transcription factors is essential for basic enhancer activity (Davidson et al., 1988; Fromental et al., 1988; Kanno et al., 1989; Macchi et al., 1989). Depending on their location within the enhancer elements, some of the specific mutations, as shown in Fig. 2(b), interfere with transcription factor binding and, consequently, abolish basic enhancer activity and transactivation (Davidson et al., 1988; Fromental et al., 1988; Kanno et al., 1989; Macchi et al., 1989; Luber et al., 1991). The results of competition assays designed to specifically titrate enhancer-binding against promoter-binding transcription factors, strongly suggested that the enhancer elements,
but not the β-globin promoter, are the targets of transactivation (data not shown). Thus, transactivation of the reporter gene is dependent on stimulation of the enhancer motifs by MHBs\(^\text{a}\) and HBx.

Following calcium phosphate-mediated transfection with transactivator and reporter plasmid, cytoplasmic RNA was prepared and hybridized to a S\(^{32}\)P-labelled, single-stranded β-globin gene-specific oligonucleotide probe. For quantification of β-globin RNA, nuclease S\(_1\) was used to trim the protected and to hydrolyse the unprotected probe molecules. After denaturing polyacrylamide gel electrophoresis of samples and autoradiography, densitometric scanning was performed to determine the relative amounts of β-globin RNA. Signal intensity ratios of at least 2:1 were considered indicative of transactivation.

The results of the nuclease S\(_1\) experiments are shown in Fig. 3 and Table 1. Plasmid pG1B mediated transactivation by MHBs\(^\text{a}\) and HBx, whereas the enhancerless pG1 was inactive. Plasmids pG1TC-II\(_{\text{WT}}\), pG1TC(II + I)\(_{\text{WT}}\) and pG1TC(II + I)\(_{\text{M5}}\), each containing an intact NF-κB element that permits binding of NF-κB (Macchi et al., 1989), efficiently mediated transactivation by MHBs\(^\text{a}\) and HBx. In contrast, plasmids pG1TC-II\(_{\text{M13}}\) pG1TC(II + I)\(_{\text{M1}}\) and pG1TC(II + I)\(_{\text{M6}}\), in which the NF-κB motif has been destroyed and, therefore, the binding of NF-κB is prohibited (Macchi et al., 1989), did not support transactivation. Plasmids pG1TC(II + I)\(_{\text{M1}}\), containing two intact AP-2 motifs (Fig. 2b), probably each of which binds AP-2 (Mitchell et al., 1987; Macchi et al., 1989), and pG1TC(II + I)\(_{\text{M6}}\), containing one intact AP-2-binding element, did not mediate transactivation. In a previous study with HBx the same result was obtained (Luber et al., 1991). Similar experiments performed with CV-1 cells (African green monkey kidney line) showed that the NF-κB element, but not the AP-2 sites, of the SV40 enhancer is involved in transactivation by MHBs\(^\text{a}\) and HBx (Luber et al., 1991 and data not shown). Taken together, our data suggest that, in Chang liver and CV-1 cells, transcription factor NF-κB, but not AP-2, is able to participate in transactivation of the TC(II + I) element of the SV40 enhancer.

We next examined the ability of the AP-1 element of the SV40 enhancer to mediate transactivation by MHBs\(^\text{a}\) and HBx. However, in Chang liver cells (Fig. 3 and Table 1) and CV-1 cells (data not shown), no transactivation was obtained with plasmid pG1SVPWT. Plasmid pG1SVPWT gave only little basic activity that was about the same as that obtained with control plasmid pG1SVP\(_{\text{M1}}\). Plasmid pG1SVP\(_{\text{M1}}\) contains a mutation in the AP-1 site (Fig. 2b) that prevents binding of factor AP-1. Apparently, the AP-1 element of the SV40 enhancer does not participate in basic enhancer function and transactivation. This finding corresponds with results obtained by Zenke et al. (1986) and Nomiyama et al. (1987) who, using several different cell lines, examined the influence of mutations within the SV40 enhancer on transcriptional activity.

In contrast to our data it was suggested by Seto et al. (1990) that, in CV-1 cells, HBx acts through the AP-1 and AP-2 sites of the SV40 enhancer. It is possible that the discrepancy between our results and the results of Seto et al., with regard to a participation of the SV40 AP-1 and AP-2 motifs in transactivation by HBx, is based on the particular structure of the reporter constructs used in the two studies.

Unlike the AP-1 motif of the SV40 enhancer, the AP-1 element of the hMT IIA enhancer, contained in pG1MTP\(_{\text{WT}}\), efficiently mediated transactivation (Fig. 3 and Table 1). The difference in the capability of the AP-1 elements of the SV40 and the hMT IIA enhancer to support transactivation might be based on their different nucleotide sequences. Although binding of AP-1 to the cognate binding site in the SV40 and the hMT IIA enhancer has been demonstrated (Angel et al., 1987; Lee et al., 1987), it is possible that distinct fos–jun combinations, each specific for one of these recognition sequences, might bind to the AP-1 elements. We hypothesize that the fos–jun pair binding to the AP-1 site in the hMT IIA enhancer mediates transactivation, whereas the fos–jun pair binding to the AP-1 site in the SV40 enhancer is unable to participate in transactivation.

Plasmid pG1GT(II + I)\(_{\text{WT}}\) clearly mediated transactivation by HBx whereas transactivation by MHBs\(^\text{a}\) was barely detectable (Fig. 3 and Table 1). The intensity of the bands resulting from cotransfections with pG1GT(II + I)\(_{\text{WT}}\) and HBx is at least ten-fold stronger than that detected with pG1GT(II + I)\(_{\text{WT}}\) and MHBs\(^\text{a}\). This is in contrast to the result obtained with pG1MTP\(_{\text{WT}}\), containing the AP-1 binding site of the hMT IIA enhancer, where transactivation by MHBs\(^\text{a}\) and HBx gave rise to about the same intensity (Fig. 3). These observations indicate that HBx transactivates the GT(II + I) element much more efficiently than MHBs\(^\text{a}\), whereas HBx and MHBs\(^\text{a}\) are equally efficient to transactivate the AP-1 motif. We assume that HBx uses a more efficient route than MHBs\(^\text{a}\) for the transactivation of GT(II + I), whereas the same activation pathway or, alternatively, equally effective pathways to transactivate the AP-1 motif are used by HBx and MHBs\(^\text{a}\). In addition to the AP-1 site of the hMT IIA enhancer, elements TC-II and TC(II + I) of the SV40 enhancer were also transactivated to about the same degree by MHBs\(^\text{a}\) and HBx (Fig. 3). Surprisingly, in cotransfections with pHBV824fs, which was used as a negative control, the activity of pG1GT(II + I)\(_{\text{WT}}\) was considerably higher than the basal level of activity obtained in experiments.
Fig. 3. Nuclease S1 analyses of RNA transcribed from pG1, pG1B and pG1 recombinants containing oligomers of the enhancer elements shown in Fig. 2(b). About 10^6 Chang liver cells were cotransfected with plasmid pVMHBs(107) (0.5 μg), pVMHBs (0.5 μg), pHBV824 (2.0 μg) or pHBV824s (2.0 μg) and reporter plasmid (1.0 μg). After 40 h of incubation, cytoplasmic RNA extracts were prepared and β-globin transcripts were quantified by nuclease S1 analysis as described previously (Koch et al., 1989). The major signal (double band) represents probe fragments of about 60 nucleotides that were protected by RNA molecules initiated at the β-globin gene promoter of the reporter plasmids. The signals of size markers loaded on the gels in separate lanes are not shown. The gels were exposed to Kodak X-OMAT AR films at -75 °C.

Earlier studies showed that the coordinate binding of TEF-1 to GT-IIC and of TEF-2 to GT-I is required for enhancer activity of the GT(IIC+I) element (Davidson et al., 1988; Fromental et al., 1988). TEF-1 and TEF-2 were originally isolated from HeLa cells (Davidson et al., 1988; Xiao et al., 1991). TEF-1 is also present in human keratinocytes where it is required for the transcription of the human papilloma virus oncogenes E6 and E7 (Ishiji et al., 1992). We assume that factors related to or identical with TEF-1 and TEF-2 facilitate transactivation via GT(IIC+I) in Chang liver cells. Destruction of the GT-IIC motif, as in pG1GT(IIC+I)M1 (Fig. 2b), interferes with transactivation. Previous experiments showed that a mutation in the GT-I motif that prevents binding of TEF-2 results in the loss of transactivation by HBx (Luber et al., 1991). Together, these observations suggest that cooperation of TEF-1 and TEF-2 is required for transactivation induced by MHBs and HBx, although only little transactivation is obtained with MHBs if compared with HBx.

Plasmid pG1Sph(II+I)WT efficiently mediated transactivation by HBx but it was unable to mediate transactivation by MHBs (Fig. 3 and Table I). Similarly to the result obtained with GT(IIC+I), it appears that, for transactivation of Sph(II+I), HBx is stimulating a pathway that cannot be effectively activated by MHBs.
Table 1. Transactivation of pG1 recombinants by MHBs and HBx in Chang liver cells

<table>
<thead>
<tr>
<th>Reporter plasmid</th>
<th>pSVMHBs&lt;sup&gt;3&lt;/sup&gt;:pSVMHBs (MHBs)</th>
<th>pHBV824:pHBV824fs (HBx)</th>
</tr>
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<tbody>
<tr>
<td>pG1</td>
<td>NDS</td>
<td>NDS</td>
</tr>
<tr>
<td>pG1B</td>
<td>4:1</td>
<td>4:1</td>
</tr>
<tr>
<td>pG1 TC-II</td>
<td>M13</td>
<td>4:1†</td>
</tr>
<tr>
<td>pG1 TC(II+I)</td>
<td>M1</td>
<td>NDS</td>
</tr>
<tr>
<td>pG1 MTP</td>
<td>M1</td>
<td>3:1</td>
</tr>
<tr>
<td>pG1 SVP</td>
<td>M1</td>
<td>NDS</td>
</tr>
<tr>
<td>pG1 GT(IIC+I)</td>
<td>M1</td>
<td>3:1</td>
</tr>
<tr>
<td>pG1 Sph(II+I)+Oct</td>
<td>M1</td>
<td>NDS</td>
</tr>
</tbody>
</table>

* Determination of relative transactivation was based on densitometric scanning of several exposures of autoradiographs from at least three experiments with two different plasmid preparations. (SD ± 20% of the means).

† The relative transactivation of pG1TC-IIWT by pHBV824 has been determined as 4:1 previously; no transactivation has been observed with pG1TC-IIM13 (Luber et al., 1991).

‡ The signal obtained with pHBV824 and pG1GT(IIC+I) was at least tenfold stronger than the signal obtained with pSVMHBs<sup>3</sup> and pG1GT(IIC+I). See text and Fig. 3.

NDS, no detectable signals.

similarity with the Sph motifs (Davidson et al., 1988). Our experiments with pHBV824, encoding HBx, demonstrated that the base changes in pG1Sph(II+I)M1 do not interfere with transactivation, whereas the base changes in pG1Sph(II+I)M2 resulted in a loss of transactivation (Fig. 3 and Table 1). This observation fully corroborates with the previous findings that the mutation in M2 but not the mutation in M1 prevents binding of TEF-1 and eliminates enhancer activity (Davidson et al., 1988; Fromental et al., 1988). The Oct motif, binding octamer transcription factors (Rosales et al., 1987), is partially overlapping with each of the two Sph elements (Fig. 2). Octamer-binding proteins are apparently not involved in transactivation by HBx, since the mutation in pG1Sph(II+I)M1, that would prevent the binding of octamer factors but not the binding of TEF-1 (Rosales et al., 1987; Davidson et al., 1988) did not interfere with transactivation (Fig. 3 and Table 1).

The essential finding of this study is that the enhancer sequences GT(IIC+I) and Sph(II+I) are efficiently transactivated only by HBx but not by MHBs. Transcriptional interference experiments have indicated that a limiting coactivator of TEF-1 is required for transcriptional competence (Xiao et al., 1991; Ishiji et al., 1992). It is presently not possible to propose a candidate target for HBx, since the cellular mechanism that leads to the activation of TEF-1 is not known. A possible interpretation of the disparate target specificity of HBx and MHBs is that HBx but not MHBs directly interacts with and thereby activates TEF-1 or its coactivator. However, in contrast to the cAMP response element-binding (CREB) protein and activating transcription factor (ATF)-2, which have been demonstrated to be present in specific protein complexes with HBx (Maguire et al., 1991), it is not known whether TEF-1 or its coactivator can associate with HBx.

We have investigated a relatively small subset of the known regulatory elements that respond to HBx and MHBs. Comparative analysis of additional target sequences of HBx and MHBs will presumably reveal further elements that are stimulated by only one of the two HBV transactivators and help to identify the regulatory pathways that are activated by either factor.

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