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DIMERIZATION OF HEPATITIS B VIRAL X PROTEIN SYNTHESIZED IN A CELL-FREE SYSTEM

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Hepatitis B viral X protein (HBx), a 17-kDa polypeptide, has been demonstrated as a trans-acting factor. In this study, we report that the HBx was able to form a dimer, a feature very similar to many well known trans-acting factors. In vitro synthesized HBx, after immunoprecipitation and analysis by SDS-PAGE, appeared as one prominent 17-kDa band (monomer) and a faint 34-kDa band (dimer). The amount of dimer increased if the sample of immunoprecipitated HBx was not treated with 2-mecaptoethanol, indicating the dimer was held together by the disulfide linkage. Dimerization of a truncated HBx established that the four cysteine residues close to the N-terminus are sufficient for the dimerization process.

Hepatitis B virus (HBV) is one of the medically important viruses because it can cause both acute and chronic hepatitis and has been linked to the formation of primary hepatocellular carcinoma in the human (1). The HBV is a DNA virus with a genome size of around 3200 bp arranged in a partial double-stranded circular form (2). Four open reading frames (ORFs), deduced from the cloned HBV-DNA sequence, are designated as pre-C/C, P, pre-S/S, and X, and encode the core antigen (HBc), DNA polymerase (P), surface antigen (HBs), and X protein (HBx), respectively (for reviews, see 3,4). Among these four viral antigens, the

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Abbreviations: aa, amino acid(s); bp, base pair(s); DTT, dithiothreitol; kDa, kilo dalton(s); 2-ME, 2-mecaptoethanol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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biological function of the HBx is least well understood. Several lines of observation have indicated that the HBx possesses a trans-activation effect on various DNA sequences, such as the enhancer element of the HBV, SV40, and RSV, the LTR of HIV, and the promoter of the β-interferon gene (5-9). The trans-activation mechanism of HBx, however, is unknown.

Elucidation of the trans-activation mechanisms of the cellular, proto-oncogene, or viral gene products has become an important goal in molecular biology. Characterization of these transcriptional regulation factors indicates that many of them are required in the form of homodimers (C/EBP, GCN4, c-myc, and c-jun) or of a heterodimer (c-jun/c-fos) to fully exert their DNA binding activity (10-14). This led us to examine whether HBx has dimerization characteristics. In this communication, we report that in vitro translated HBx was able to form a dimer via disulfide linkages.

MATERIALS AND METHODS

Enzymes and chemicals. Restriction enzymes, T4 DNA ligase, and SP6 RNA polymerase were purchased from New England Biolabs (Beverly, Ma., USA) or Promega Biotec (Madison, Wi., USA) and were used according to the prescribed reaction conditions. The rabbit reticulocyte lysate was purchased from Promega Biotec and [^{35}S]-methionine from Amersham (Aylesbury, Buckinghamshire, England).

Bacterial strains and Plasmids. E. coli strain JM103 and DH5α were used for bacterial transformation and plasmid amplification. The plasmid pTWL1 supplying the HBV-DNA was described previously (15). The pGEM1 was purchased from Promega.

In vitro Transcription and Translation. The plasmid used for in vitro transcription was purified by cesium chloride banding then linearized with SmaI or HincII. The template DNA (5 μg) was added into a reaction mixture containing 40 mM Tris hydrochloride (pH 7.5), 6 mM $MgCl_2$, 10 mM DTT, 40 U RNasin, 0.5 mM of ATP, CTP, and UTP, 0.25 mM GTP, 0.5 mM GpppG, and 20 U SP6 RNA polymerase in a total volume of 50 μl. After 1-2 h incubation at 40° C and digestion by 5 U DNase, the in vitro synthesized RNA was phenol-extracted and the integrity analyzed in a formaldehyde-agarose gel. For in vitro translation, the protocol from the supplier was followed, i.e., the protein synthesis was carried out in a volume of 50 μl supplemented with 50 μCi of [^{35}S]-methionine. The in vitro translated proteins, either directly or after immunoprecipitation, were analyzed by SDS-PAGE.

Immunoprecipitation and Gel Electrophoresis. Antibodies against the M13 Gene II-HBx fusion protein (16) or against synthetic polypeptides of HBx (gifts from Dr. M. Feitelson) were used to immunoprecipitate the in vitro translation product. The immunoprecipitated proteins were dissolved in sample buffer with or without 2-mecaptoethanol (2-ME). SDS-PAGE was carried out on 12 or 15% separating slab gels in the discontinuous system of Laemmli (17). Two dimensional SDS-PAGE was performed by running the sample without 2-ME treatment in the first dimension gel and by following the procedure of O'Farrell (18) in the second dimension gel. The gels were fixed and dried, and exposed to a Fuji X-ray film at -70° C.

RESULTS AND DISCUSSION

A 618-bp NcoI-BglII fragment of HBV-DNA, containing the entire X gene and part of the pre-C/C gene, was cloned downstream from the promoter of the SP6 RNA polymerase in the pGEM1 to produce the plasmid pSP6-X1 (Fig. 1A). The pSP6-X1 was completely linearized by either SmaI or HincII digestion (Fig. 1A) and subjected to an in vitro transcription system using SP6 RNA polymerase to generate two unique lengths of RNAs, XRNA1 or XRNA2, respectively (Fig. 1B). Both RNAs start with the same 5'-end sequence of GAAUACAAGCUC in front of the translation initiation codon of HBx. They terminate, however, at different 3'-ends to encode a 154-aa authentic HBx (17 kDa) or a 103-aa truncated form of HBx (12 kDa) (Fig. 1A).

Fig. 2 represents one serial SDS-PAGE analysis of in vitro translation products of XRNA1. Such an autoradiograph routinely showed the presence of one prominent 17-kDa band in the 2-ME treated sample and an additional 34-kDa band, which appeared in the same translation sample without 2-ME treatment (Fig. 2A, lane 2 vs. lane 4). Immunoprecipitation experiments insured that the major XRNA1 translation products were indeed the HBx, which could also appear in high-molecular-weight forms in the absence of 2-ME treatment conditions (see Fig. 2B, lane 4). Quantitative differences in the various forms of HBx were noted, however, between those of anti-X antibody precipitated and non-

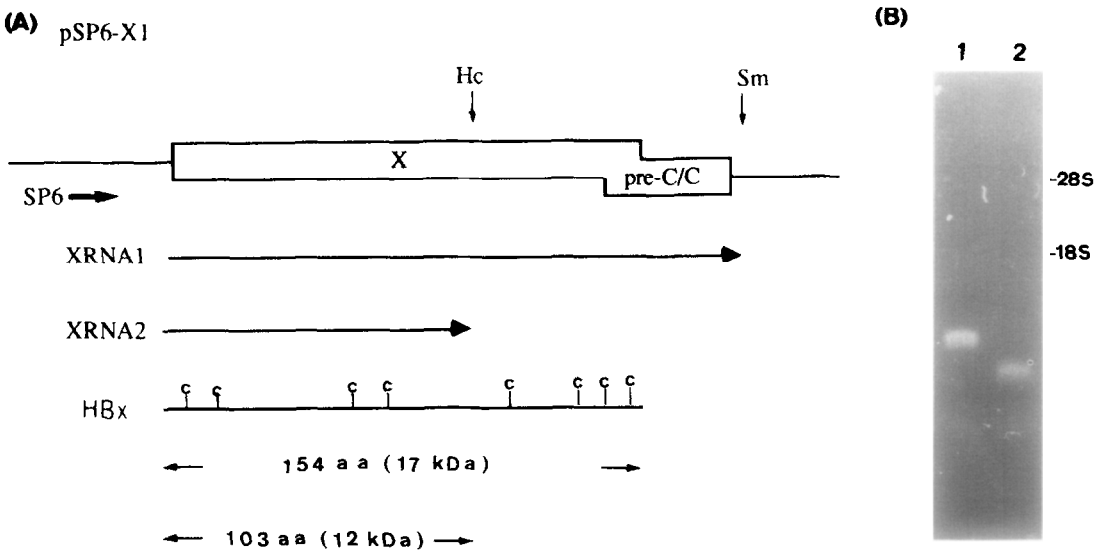

Fig. 1 Partial physical map of pSP6-X1 and two transcribed RNAs. (A) The open box, containing the HBV X and pre-C/C genes overlapped by 25 bp in the junction, is flanked by two thin lines representing a partial sequence of pGEM1. The symbol of SP6 with arrow indicates the location of the promoter of the SP6 RNA polymerase and the direction of transcription in the pSP6-X1. Vertical arrows on the top show specific restriction sites used for linearization of pSP6-X1; Hc:HincII, Sm:SmaI. Two RNAs (XRNA1 and XRNA2) generated from the SmaI- or HincII-digested DNA template are shown by long arrows. The sizes of authentic and truncated HBx translated by XRNA1 and XRNA2 are bracketed by arrows, indicating numbers of amino acids and molecular weight. Eight c depicted on the thin line, corresponding to their relative positions (7, 17, 61, 69, 116, 137, 143, and 148) on the HBx, represent conservative cysteine residues in 10 HBV isolates (19). (B) A formaldehyde-agarose gel shows the integrity of RNAs synthesized in vitro, lane 1: XRNA1 and lane 2: XRNA2. The sizes of RNA, 18S and 28S, are marked on the right.

immunoprecipitated samples, even though they were synthesized in the same batch, in the same condition of absence of 2-ME, and subjected to the same analysis by SDS-PAGE (compare lane 4 of Fig. 2A and 2B).

To determine whether the high-molecular-weight forms of HBx in the absence of 2-ME represent a dimer and an oligomer of HBx, as suggested by their migration positions on the gel, we analyzed

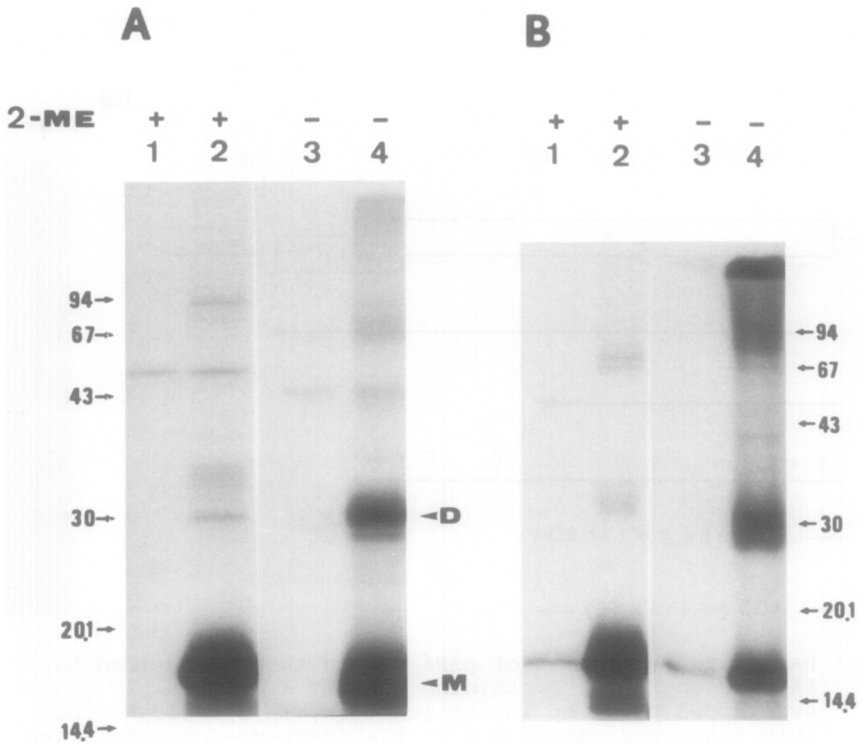

Fig. 2 Analysis of in vitro translated HBx by SDS-PAGE in the presence or absence of 2-ME. (A) Protein patterns of in vitro translation with minus RNA (lane 1, 3) or XRNA1 (lane 2, 4) and analysis in the presence of (lane 1, 2) and absence (lane 3, 4) of 2-ME. (B) The in vitro translated HBx immunoprecipitated by a normal rabbit serum (lane 1, 3) or anti-X antibodies (lane 2, 4) and analyzed in the presence (lane 1, 2) and absence (lane 3, 4) of 2-ME. M and D indicate the positions of the monomer and dimer forms of HBx. The numbers at both margins of the gels are protein molecular weight standards.

the HBx by the two dimensional SDS-PAGE technique described in the Methods section. Results shown in Fig. 3 indicate that the XRNA1 translation products, under the non-reducing condition, were present as multiple forms in the first dimensional gel (horizontal gels on the top). After the 2-ME treatment, the 34-kDa proteins were converted predominantly into a 17-kDa form in the second dimensional gel, suggesting that the 34-kDa protein is indeed the dimer of HBx held together by disulfide linkages.

To further demonstrate the dimerization capability of HBx and to identify those cysteine residues of HBx involved in the

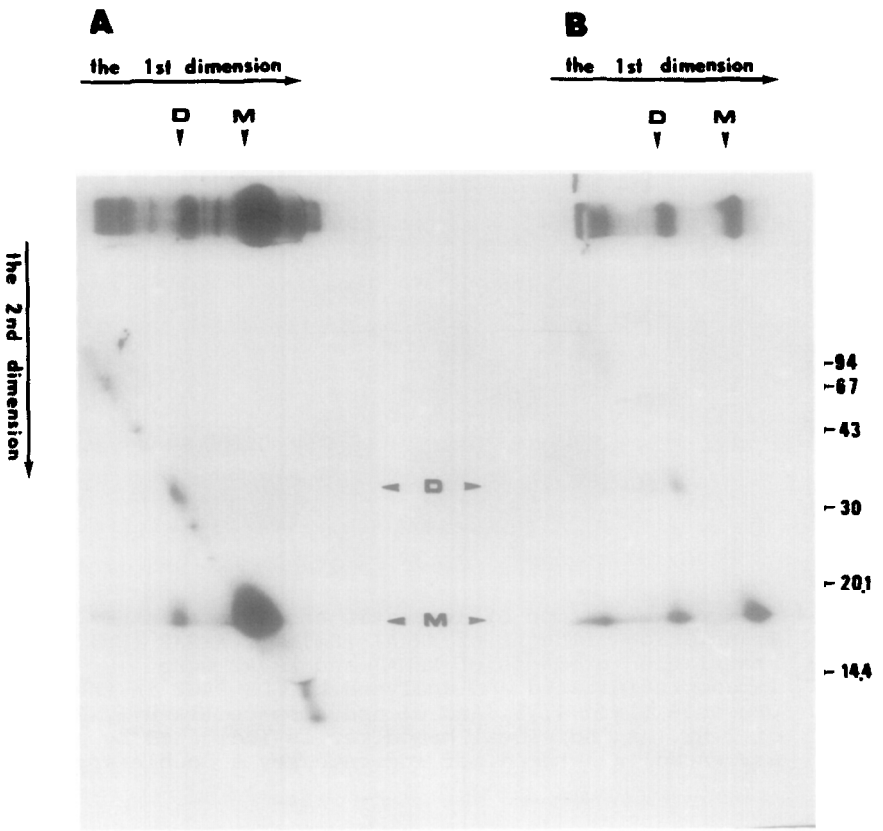

Fig. 3 Two dimensional SDS-PAGE of HBx. The horizontal arrow and gel on the top represent the first dimension gel separating the proteins without 2-ME treatment; the vertical arrow indicates the second dimension of reducing gel. (A) Proteins not immunoprecipitated, and (B) immunoprecipitated by anti-X antibodies. Other legends as indicated in Fig. 2.

dimer formation, a truncated HBx, translated from the XRNA2, was analyzed by SDS-PAGE in both reducing and non-reducing conditions. Results demonstrated that the 12-kDa (truncated) HBx was still able to form a dimer (24 kDa) in the absence of 2-ME (Fig. 4, lane 2 vs. lane 5). Moreover, the products of co-translation of XRNA1 and XRNA2, as analyzed by SDS-PAGE in the absence of 2-ME, appeared as a presumptive heterodimer of authentic and truncated HBx and also a homodimer of authentic and truncated HBx (Fig. 4 lane 3). These dimers with a binomial subunit distribution (one 12-12 kDa homodimer: two 12-17 kDa

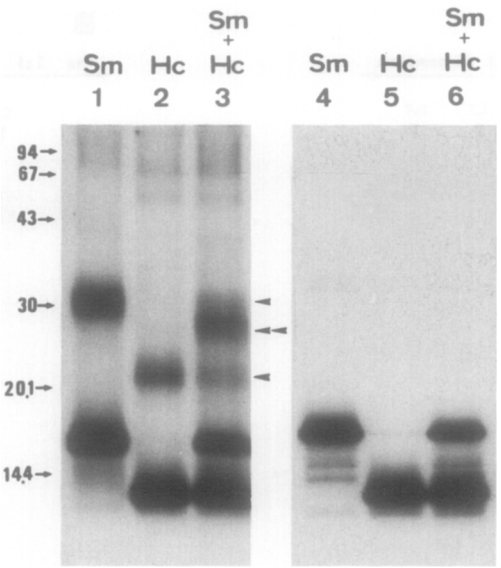

Fig. 4 Heterodimerization of authentic and truncated HBx. Translation products of XRNA1 (Sm) and XRNA2 (Hc) and co-translation products of XRNA1 and XRNA2 were immunoprecipitated and analyzed by SDS-PAGE in the presence (lane 4, 5, and 6) and absence (lane 1, 2, and 3) of 2-ME. An individual homodimer is indicated by a single-arrowhead; a heterodimer indicated by a double-arrowhead.

heterodimer: one 17-17 kDa homodimer) were consistently observed. Thus these observations have led us to conclude that the cysteine residues at positions 7, 17, 61, and 69 (Fig. 1A, 19) are involved in the HBx dimerization process.

When the amino acid sequences of HBx were re-examined, four lysine residues and one alanine were found at positions 9, 16, 30, 37, and 23 (19) in an arrangement similar to the leucine zipper motif as reported by Landschulz et al. (20). Such a structural arrangement may also contribute to dimer formation of HBx. Four different structural motifs have been previously identified in the dimerization of trans-activation factors: (i) helix-turn-helix (λ repressor, 21), (ii) helix-loop-helix (kE2, 22), (iii) metal linkage (Tat of HIV, 23), and (iv) leucine zipper (c-jun/c-fos, 13,14,20). In this study, a fifth mechanism related to the dimerization of HBx has been identified, that

associated with a disulfide linkage. Whether the dimerization of HBx is required for its trans-activation function, however, remains to be tested.

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REFERENCES

1. Beasley, R.P., Hwang, L.Y., Lin, C.C., and Chien, C.S. (1981) Lancet 2:1129-1132.
2. Gust, I.D., Burrell, C.J., Coulepis, A.G., Robinson, W.S., and Zuckerman, A.J. (1986) Intervirology 25:14-29.
3. Tiollais, P., Pourcel, C., and Dejean, A. (1985) Nature 317:489-495.
4. Ganem, D., and Varmus, H.E. (1987) Ann. Rev. Biochem. 56:651-693.
5. Twu, J.-S., and Schlomer, R.H. (1987) J. Virol. 61:3448-3453.
6. Spandau, D.F., and Lee, C.-H. (1988) J. Virol. 62:427-434.
7. Seto, E., Yen, T.S.B., Peterlin, B.M., and Ou, J.-H. (1988) Proc. Natl. Acad. Sci. (USA) 85:8286-8290.
8. Twu, J.-S., and Robinson, W.S. (1989) Proc. Natl. Sci. Acad. (USA) 86:2046-2050.
9. Siddqui, A., Gaynor, R., Srinivasan, A., Mapoles, J., and Farr, R.W. (1989) Virology 169:479-484.
10. Landschulz, W.H., Johnson, P.F., and McKnight, S.L. (1989) Science 243:1681-1688.
11. Hope, I.A., and Struhl, K. (1987) EMBO. J. 6:2781-2784.
12. Dang, C.V., McGuire, M., Buckmire, M., and Lee, W.M.F. (1989) Nature 337:664-666.
13. Nakabeppu, Y., Ryder, K., and Nathans, D. (1988) Cell 55:907-915.
14. Halazonetis, T.D., Georgopoulos, K., Greenberg, M.E., and Leder, P. (1988) Cell 55:917-924
15. Lo, S.J., Lee, Y.-H.W., Chiou, J.-S., Ting, L.-P., Liu, W.-T., and Choo, K.-B. (1985) Biochem. Biophys. Res. Commun. 129:797-803.
16. Chen, M.-L., Lee, Y.-H.W., and Lo, S.J. (1988) Gene 62:315-321.
17. Laemmli, U.K. (1970) Nature 227:680-685.
18. O'Farrell, P.H. (1975) J. Biol. Chem. 250:4007-4021.
19. Lo, S.J., Chien, M.-L., and Lee, Y.-H.W. (1988) Virology 167:289-292.
20. Landschulz, W.H., Johnson, P.F., and McKnight, S.L. (1988) Science 240:1759-1764.
21. Pabo, C.O., and Lewis, M. (1982) Nature 298:443-447.
22. Murre, C., McCaw, P.S., and Baltimore, D. (1989) Cell 56:777-783.
23. Frankel, A.D., Bredt, D.S., and Pabo, C.O. (1988) Science 240:70-73.