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Transcription of dbpA, a Y box binding protein, is positively regulated by E2F1: implications in hepatocarcinogenesis

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Abstract

Human hepatocellular carcinoma is one of the most common cancers in the world. We previously showed that dbpA, a member of the Y box family of proteins, could accelerate the process of inflammation-induced hepatocarcinogenesis, and that dbpA is more abundantly expressed in hepatocellular carcinoma than in non-tumorous tissue. In this study, to clarify the mechanism by which expression of dbpA is enhanced in the proliferative state, we examined the transcriptional activity of the dbpA promoter region. We focused on the sequence 5′-TTTGGGGC-3′ (−8 to −1 in the promoter region) resembling the E2F binding site (one base mismatch, TFSEARCH score 86.2). By overexpressing E2F1 in Huh-7 cells, transcriptional activity of dbpA was significantly increased, and this increase was abolished by mutating or deleting this sequence. Thus, expression of dbpA was positively regulated by E2F1, suggesting that one of the effects of E2F1 on cell proliferation might be mediated by dbpA at the carcinogenesis step.

Keywords: dbpA; E2F1; DP-1; Hepatocarcinogenesis

DNA binding protein A (DbpA) [1] belongs to the Y box binding protein family. This family is characterized by the common DNA/RNA binding region called the "cold shock domain," which is conserved in species ranging from bacteria to mammals [2]. The Y box binding proteins have multi-functions, such as the regulation of transcription and translation [3], and are thought to play a role in cell proliferation or transformation. The nuclear expression of YB-1, the prototype member of this family, is reported to be associated with more advanced stages of malignant diseases, such as breast cancer [4], non-small lung cancer [5], thyroid cancer [6], and colorectal cancer [7]. The function of Y box binding proteins is suspected to be important for the progression of cancer, although at present, it is still unclear how they are involved in carcinogenesis at the molecular level.

Human hepatocellular carcinoma (HCC) is one of the most common cancers in the world. Epidemiologically, chronic infection with hepatitis B virus or hepatitis C virus is strongly linked to the development of HCC. However, not all viral carriers develop HCC, and HCC is rare in asymptomatic viral carriers. When inflammation is more severe and of longer duration, HCC develops more frequently. Therefore, chronic inflammation may have a causative role in hepatocarcinogenesis, but its molecular mechanisms are still unknown. We previously reported that dbpA, a member of the Y box binding proteins, could accelerate the process of inflammation-induced hepatocarcinogenesis [8]. The expression of dbpA was more abundant in HCC than in adjacent non-tumorous tissue, and thus, like

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YB-1, dbpA appears to be a proliferation-associated protein.

In this report, in order to clarify why expression of dbpA is enhanced in the proliferative cells, we studied the transcriptional regulatory region of dbpA. Kudo et al. [9] previously reported the core promoter sequence of dbpA. A search of the public databases (TRANSFAC [10] and TFSEARCH [http://www.rwcp.or.jp/papia/]) showed that within this region the sequence 5′-TTT GGGGC-3′ (−8 to −1 in the promoter region) is highly similar to the E2F binding site 5′-TTT(G/C)GCGC-3′ (TFSEARCH score 86.2). Recently, E2F1 was also reported to be involved in hepatocarcinogenesis in a mouse model [11]. Therefore, we concentrated our attention on the significance of E2F1 in the transcriptional control of dbpA. By using the luciferase assay, we examined the effect of E2F1 on the transcriptional activity of the dbpA promoter region. By the successive deletion of this sequence in the luciferase vector, the transcriptional activity decreased. When E2F1 was overexpressed in Huh-7, the transcriptional activity increased significantly, but this increase was abolished when the sequence in the luciferase vector was mutated or deleted. Thus, E2F1 appears to positively regulate the transcription of dbpA. We also demonstrated that the expression of dbpA and DP-1 (the heterodimerization partner of E2F1) is synchronized within the regenerating liver of mice after partial hepatectomy. DbpA appears to be one of the downstream targets of E2F1/DP-1 signaling, and it may play a role in promoting cell proliferation or transformation.

Materials and methods

Plasmid construction. The firefly luciferase expression vector was made by insertion of the synthesized DNA corresponding to the dbpA promoter region into pGL3-Basic vector (Promega). The DNAs were synthesized so that they contained the *Mlu*I and *Bgl*II recognition site at each end after annealing. They were digested with *Mlu*I and *Bgl*II, and ligated to the *Mlu*I–*Bgl*II digested pGL3-Basic vector. The synthetic DNAs covering −17 to +106 relative to the transcription start point (123 mer), −4 to +106 (110 mer), and +1 to +106 (106 mer) were inserted into pGL3-Basic vector (Fig. 2A). The 123 mer DNA contained the 8 mer sequence resembling the E2F binding site, the 110 mer DNA contained a 4 mer sequence corresponding to half of the 8 mer sequence resembling the E2F binding site, and the 106 mer DNA did not contain the 8 mer sequence. As shown in Fig. 3A, we also synthesized five oligomers covering −17 to +66, named Wild, M1, M2, M3, and M4, with internal sequence modifications. "Wild" contained the wild type sequence resembling the E2F1 binding site. In M1, that sequence was replaced with the sequence 5′-TTTCGCGC-3′ which perfectly matches to the E2F binding site. In M2 and M3, the sequence was changed as shown in Fig. 3. In M4, the whole sequence resembling the E2F binding site was deleted. These five oligomers were inserted into pGL3-Basic vector. An intact pGL3-Basic vector was used as a mock reporter vector. The human E2F1 expression vector was a kind gift from Dr. M. Hatakeyama (Division of Molecular Oncology, Hokkaido University, Sapporo, Japan), and consisted of human E2F1 cDNA inserted in pcDNA3 vector (Invitrogen). An intact pcDNA3.1 (+) vector (Invitrogen) was used as a negative control. In pcDNA3 and pcDNA3.1 (+) vector, the transcription was driven by cytomegalovirus (CMV) immediate early enhancer/promoter. As internal controls for the dual luciferase assay, pRL-TK (Toyo Ink, Tokyo) expressing *Renilla* luciferase driven by herpes simplex virus thymidine-kinase (HSV-TK) promoter, or pRL-SV40 (Toyo Ink) expressing *Renilla* luciferase driven by SV40 early enhancer/promoter was used.

Transient transfection and luciferase assay. Huh-7 cell, derived from human hepatocellular carcinoma, was purchased from Riken Cell Bank (Tsukuba, Japan). Huh-7 cells were maintained in DMEM (Gibco-BRL) supplemented with 10% FCS, maintained in 5% CO_2 at 37 °C. Transient transfection of Huh-7 was performed by the lipofection method [12] using Fugene 6 (Roche). Huh-7 cells (3×10^5 cells) were seeded onto a 35 mm plate, and 12 h later, the medium was discarded and replaced. Another 12 h later, transfection was performed and the cells were harvested 24 h later. To assay luciferase activity [13] in Huh-7 cells without overexpression of E2F1, 1 μg of a reporter vector or a mock reporter vector (pGL3-Basic), 10 ng pRL-TK, and 6 μL Fugene 6 reagent were added per 35 mm plate. To assay the activity in Huh-7 cells with overexpression of E2F1, 1 μg reporter vector or mock reporter vector (pGL3-Basic), 1 μg human E2F1 expression vector or a negative control (pcDNA3.1 (+)), 20 ng pRL-TK, and 12 μL Fugene 6 were added per 35 mm plate. Luciferase activity was measured with a PicaGene dual assay kit (Toyo Ink) and an automatic luminometer (Lumat LB953) (EG & G Berthold, Bad Wildbad, Germany) according to the manufacturer's instructions. Measured values were used for the calculation of relative luciferase activity as follows; relative activity = [firefly activity/*Renilla* activity] of a reporter vector divided by [firefly activity/*Renilla* activity] of a mock reporter vector (pGL3-Basic). The experiments were repeated six times, and the results were expressed as means ± SEM. Statistical analysis was performed by Mann–Whitney *U* test using StatView ver. 4.0 (Abacus Concepts) statistical analysis software.

Hepatectomy of mice. Ten-week-old male C57BL/6 mice ($n = 15$) were purchased from Clea Japan (Tokyo, Japan). The mice were anesthetized with pentobarbital (30 mg/kg i.p.). Twelve mice were subjected to two-thirds hepatectomy and were sacrificed 12, 24, 36, and 48 h ($n = 3$ per group) after hepatectomy. A control group ($n = 3$) was subjected to a sham operation, in which the liver was pushed out through the abdominal incision and then returned, and then sacrificed 48 h later. All liver samples were stored at −80 °C until use.

RNA isolation and Northern blotting analysis. Total RNA was isolated from the liver by the acid guanidinium thiocyanate–phenol–chloroform method [14], using Trizol (Invitrogen). Total RNA (10 μg) was run on 1.0% agarose gels in 20 mM Mops (pH 7.0), 15 mM sodium acetate, 1 mM EDTA, and 10% formamide. After staining of the gels with 1 μg/mL ethidium bromide and photographing of the 28S and 18S RNA, the resolved RNA was transferred to a nylon membrane, Biodyne B (Pall Corp.) in 20× SSC (3 M NaCl, 300 mM sodium citrate). Hybridization was performed overnight at 65 °C in 1% BSA, 7% SDS, 1 mM EDTA, and 200 mM sodium phosphate (pH 7.2) containing 1×10^6 cpm/mL ^{32}P-labeled probe which was prepared by random-prime labeling using Prime-It II (Stratagene), of cDNAs of dbpA, E2F1, and DP-1. Membranes were washed at 65 °C in 0.1% SDS, 1× SSC (150 mM NaCl, 15 mM sodium citrate) and exposed to Kodak XAR-5 film at −40 °C [15].

Results

Decreased transcriptional activity of dbpA promoter region without the sequences resembling E2F binding site

As shown in Fig. 1, the sequence 5′-TTTGGGGC-3′ (−8 to −1) resembling the E2F binding element 5′-

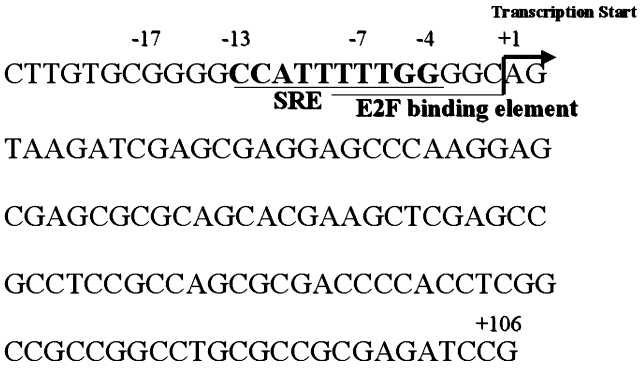

Fig. 1. Nucleotide sequence of the human dbpA promoter region [9]. The sequence between −24 and +106 relative to the transcription start site (indicated by arrow) is shown. The sequence resembling SRE is shown with bold letters, and that resembling the E2F binding site is underlined.

TTT(G/C)GCGC-3′ exists in the dbpA promoter region. In order to examine whether this sequence is important for the transcriptional activity of this promoter, we prepared reporter vectors containing this region with successive deletions, shown in Fig. 2A, and assayed their luciferase activity. Luciferase activity was highest with the vector containing the (−17 to +106) region, in which the 8-base sequence was preserved. The promoter (−4 to +106) that contained half of this sequence showed a significant decrease in activity as compared with the promoter (−17 to +106). The transcription activity of the promoter (+1 to −106) lacking this sequence was the lowest (Fig. 2B). We repeated the assay with the transient overexpression of E2F1. Under those conditions, the effect of the sequence on transcription was more pronounced, as shown in Fig. 2C. These data showed that the sequence resembling the E2F binding element and E2F1 are important for the transcriptional activity of this promoter.

Positive effect of E2F1 on the transcriptional activity of dbpA promoter

We performed additional luciferase assays with reporter vectors containing the dbpA promoter region with a nucleotide change within the sequence resembling the E2F binding site. Fig. 3A shows a schematic representation of insert DNA in the reporter vector. As shown in Fig. 3B, the overexpression of E2F1 significantly enhanced the transcriptional activity of the wild type sequence. When the wild type sequence was changed to the consensus E2F binding motif (M1), the effect of overexpressed E2F1 slightly increased. However, mutation or deletion of this sequence, as in M2, M3, and M4, completely abolished the effect of overexpressed E2F1. We carried out similar assays using pRL-SV40 instead of pRL-TK, as the control vector, and the result was essentially same as that with pRL-TK

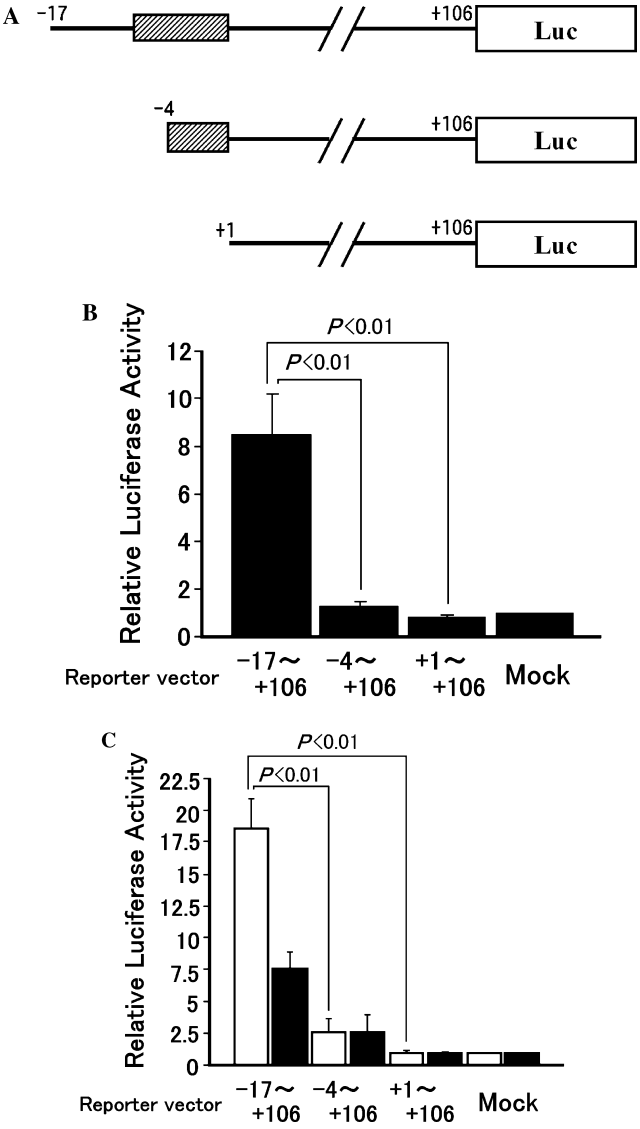

Fig. 2. Decreased transcriptional activity of the dbpA promoter region without the sequence resembling the E2F binding site. (A) Schematic representation of reporters with DNA insertion. The sequence resembling the E2F binding element is represented by the hatched box. The firefly luciferase reporter gene is represented by the open box. (B) Effect of the sequence resembling the E2F binding element on transcriptional activity. Data are shown as means ± SEM from six experiments. (C) Effect of E2F1 on the promoter region with and without the sequence resembling the E2F1 binding element. White bar: transcriptional activity when E2F1 was overexpressed. Black bar: activity when mock vector was transfected.

(data not shown). These results confirmed that E2F1 and E2F binding sites are involved in the positive regulation of the transcriptional activity of dbpA promoter.

Transcriptional changes of dbpA, E2F-1, and DP-1 in the mouse regenerating liver

Previously, we reported the enhanced expression of dbpA in the regenerating liver. Here we studied the rela-

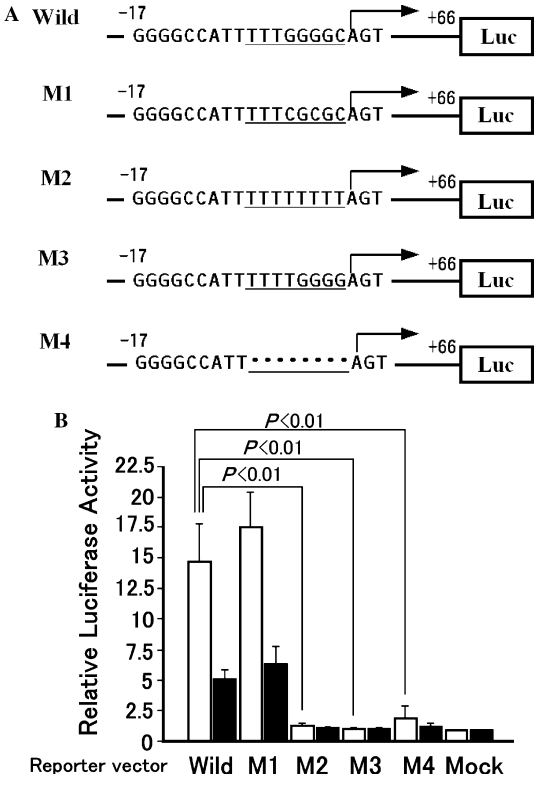

Fig. 3. The positive effect of E2F1 on transcriptional activity of the dbpA promoter. (A) Schematic representation of reporters. The transcription start site is indicated by an arrow (see Materials and methods). (B) Effects of E2F1 overexpression on the wild type and mutated sequence in the promoter region. White bar: transcriptional activity when E2F1 was overexpressed. Black bar: activity when mock vector was transfected.

tionship between the transcription of dbpA, E2F1, and DP-1 (which is a partner of E2F1 heterodimerization) in the mouse regenerating liver. As shown in Fig. 4, all three transcripts were most abundantly expressed 36 h after hepatectomy. The expression of dbpA was mildly enhanced 12 h after hepatectomy, slightly decreased but higher than the control at 24 h, and clearly enhanced at 36 h (second panel in Fig. 4). The expression of two E2F1 transcripts (2.2 and 2.7 kb) [16] was not enhanced at 12 h, and their levels were similar to those of the control. The expression of E2F1 started to increase after 24 h. The expression pattern of E2F1 did not parallel that of dbpA (third panel in Fig. 4). Since the levels were low, we used RT-PCR to confirm the existence of E2F1 transcripts both in the control and at 12 h after hepatectomy (data not shown). As shown in the bottom panel of Fig. 4, the expression pattern of DP-1 was very similar to that of dbpA, i.e., mild increase at 12 h and a clear increase at 36 h. The expression of dbpA and DP-1 seemed to be synchronized.

Discussion

The frequency of human HCC is dependent on the severity and duration of chronic inflammation of the liver, regardless of its etiology. We previously reported that dbpA, a member of the Y box binding protein family, could accelerate inflammation-induced hepatocarcinogenesis [8]. The finding of enhanced expression of dbpA in human HCC [8] and in regenerating mouse liver (Fig. 4) suggested that dbpA is a proliferation-associated protein, like YB-1, the prototype member of the same family [3,4,17]. To determine the mechanism by which expression of dbpA is enhanced in the proliferative state, we examined the transcriptional regulation of dbpA. Kudo et al. [9] reported that the transcriptional control region of dbpA existed within position −17 to +70 relative to the transcription start point. They also described the sequence 5′-CCATTTTTGG-3′ (−13 to −3) as being similar to serum responsive element (SRE) 5′-CCATATTAGG-3′. A motif search using

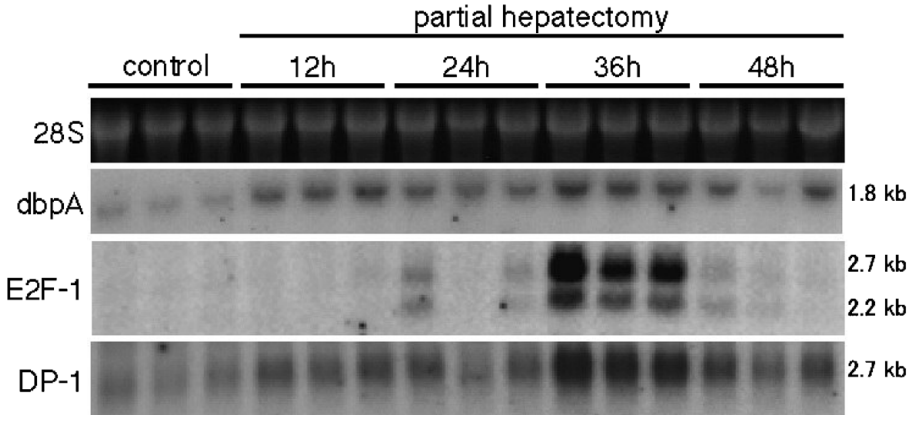

Fig. 4. Enhanced expression of dbpA, E2F1, and dbpA in the regenerating liver. Total RNA (10 μg) was extracted from the liver of sham-operated mice (control), and mice at 12, 24, 36, and 48 h after hepatectomy ($n = 3$ per group) and run on the respective lanes. Top panel shows ethidium bromide-stained 28S ribosomal RNA in the agarose gel used for Northern blotting to detect the dbpA transcript shown the in second panel. As probes for Northern blotting, cDNAs of dbpA (second panel), E2F1 (third panel), and DP-1 (fourth panel) were used.

TFSEARCH and TRANSFAC [10] revealed the sequence 5′-TTTGGGGC-3′ (−8 to −1) to be similar to the E2F binding site 5′-TTT(G/C)GCGC-3′ as indicated by the higher TFSEARCH score (86.2 for E2F binding site vs 83.6 for SRE). Although SRF and E2F are both transcriptional factors associated with cell proliferation, we focused on the E2F binding site because several studies have reported the importance of E2F1 in the development of HCC [18,19]. Pascale et al. [20] reported the enhanced expression of E2F1 in HCC of rats treated with diethylnitrosoamine. Conner et al. [11] reported the development of HCC in E2F1 transgenic mice. Yasui et al. [21] reported that E2F1 was overexpressed in human HCC as compared with adjacent non-tumorous liver tissue, and that the overexpression occurs at an early stage of hepatocarcinogenesis [21]. Based on these findings, we examined the effect of E2F1 on the transcription of dbpA and showed that E2F1 positively regulated the transcription of dbpA. Because our present work focused on E2F1, we cannot exclude the possibility that other transcriptional factors affect dbpA transcription. Based on the results of in vitro experiments (shown in Figs. 2 and 3), we reasoned that the in vivo upregulation of dbpA in the cellular proliferative state might also be mediated by the increased activity of E2F1. We examined whether the expression level of dbpA was related to that of E2F1 or DP-1 in the mouse regenerating liver (Fig. 4). The results showed that the peaks of expression of three transcripts were seen at the same time (36 h after hepatectomy), and this time almost coincided with the time of maximum DNA synthesis, which is reported to be 35 h after hepatectomy in C57BL/6 and C3H mice [26]. These results indicated that all three transcripts were most abundantly expressed when hepatocytes were regenerating most vigorously. In the early stage following hepatectomy, the expression patterns of the three transcripts differed. At 12 h after hepatectomy, the expression of dbpA was enhanced but that of E2F1 was not, indicating that the expression patterns of dbpA and E2F1 were not paralleled. Instead, the expression of dbpA and DP-1 seemed to be well synchronized, i.e., both showing mild increases at 12 h, slight decreases at 24 h, and a more pronounced increase at 36 h. DP-1 is the partner of E2F1 heterodimerization, which can lead to a drastic increase in the specific DNA binding activity of E2F1 [22–24]. The enhanced expression of DP-1 is associated with the progression of HCC, and the activity of E2F1 seems to be controlled rather by the posttranslational conditions of E2F1, such as binding with DP-1, than by the transcriptional regulation of E2F1 itself [21,25]. The results of our hepatectomy experiments suggest that, at earlier time points, the expression of dbpA may be upregulated by the enhanced expression of DP-1, by accelerating the formation of E2F1/DP-1 complex. It is also possible that another unknown protein regulates the expression of dbpA at earlier time points. At 24, 36, and 48 h, the expressional changes of all three transcripts seemed to be similar, suggesting that the expression of dbpA at these time points may be upregulated by the enhanced expression of both E2F1 and DP-1. The regulation of dbpA expression at early time points (control and 12 h) and at later time points (24, 36, and 48 h) seemed to be different. Expression of dbpA and DP-1 showed two peaks, the first at 12 h and the second at 36 h, and at present we do not know why two peaks appear before the time of maximum DNA synthesis. E2F1 activity was originally thought to be necessary for cell proliferation, for the transition from G1 to S phase in the cell cycle [27,28]. E2F1 was later shown to be involved not only in cell proliferation but also in apoptosis [29–31], and now E2F1 is considered to act either as an oncoprotein [32] or as a tumor suppressor protein [33]. Furthermore, E2F1 has been reported to regulate not only the transcription of cell cycle-related genes, but also that of cell cycle-independent genes [34]. Thus, the diverse roles of E2F1 are recently becoming more evident. In our work, however, E2F1 enhanced the transcription of dbpA, and the expression of dbpA is clearly enhanced in the proliferative cells. According to our earlier hypothesis [8], dbpA may play a causative role in inflammation-induced hepatocarcinogenesis, and we showed the transcription of this gene was positively regulated by E2F1, which has also been implicated as a causal factor in hepatocarcinogenesis. DbpA may exert its effect on cell proliferation or transformation, as one of the downstream targets of E2F1. This mechanism, mediated by E2F1 and dbpA, may be involved in not only the development of HCC but also that of cancers in other organs.

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