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Different Protein-Binding Patterns in the P3 Promoter Region of the Human Insulin-like Growth Factor II Gene in the Human Liver Cirrhosis and Hepatocellular Carcinoma Tissues

The P3 promoter of the human insulin-like growth factor II (IGF-II) is the major IGF-II promoter in fetal liver (FL) and hepatocellular carcinoma (HCC). However, little information is available on the transcriptional factors (TFs) controlling IGF-II gene expression in human liver cirrhosis (LC) and HCC tissues. To evaluate the protein-binding patterns in the P3 promoter region, we performed electromobility shift assay (EMSA) and DNase I footprinting assay using nuclear extracts from human FL, LC and HCC tissues. EMSA showed considerable differences in binding patterns of proteins to P3 promoter region according to different nuclear extracts used in this study. By footprinting assay, eight footprints were observed in extracts. In addition, LC extract showed two specific binding at L1 [−80: +30] and L2 [−126: −80] regions, and HCC showed two specific binding at H1 [−176: −120] and H2 [−210: −177] as well as two liver specific binding (L1 and L2). Footprinting after immunoprecipitation indicates that Egr1, Egr2 and Sp1 could bind to P3 promoter directly, while c-jun and c-fos could not bind to these region directly. Further study is required to determine the function of these proteins.

Key Words : *Insulin like growth factor II (IGF-II); Liver cirrhosis; Hepatocellular carcnoma; Electromobility shift assay; Footprinting assay*

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors, particularly in certain areas endemic for hepatitis B virus infection (1) and usually develops from chronic hepatitis and liver cirrhosis. However, the molecular mechanisms of hepatocarcinogenesis from chronic hepatitis and liver cirrhosis to HCC have not yet been clearly defined, although it has been reported that a variety of growth factors might be involved in this process.

We previously reported that insulin like growth factor II (IGF-II) may play a role in the development of HCC (2). It has been known that IGF-II is required for normal pre-natal growth of the liver in rodents and humans (3, 4) and has an angiogenic effect in experimental animals (5). The human IGF-II gene is a complex transcription unit containing four different promoters (P1-P4) and two polyadenylation signal, leading to the formation of multiple IGF-II mRNA (6). The regulation of expression of human IGF-II gene is controlled by an intricate mechanism that enables the synthesis of this factor under various physiological conditions. The four promoters can be activated in a development-dependent and tissue-dependent manner (7). Analysis of IGF-II mRNA has shown that promoters P2, P3 and P4 are used with different activities in many fetal and adult nonhepatic tissues. In adult liver, promoter P1 is activated and promoter P2, P3 and P4 are completely shut off. The human IGF-II promoter P3 is used in many fetal tissues and is highly active in several tumors, suggesting autocrine effects of IGF-II in tumor progression. Sussenbach et al. have extensively characterized the sequence of P3 and P4 and suggested (8-11) that the Egr family, which bind early growth response element (Egr-1), GCGGGGGCG, might play an essential role in the transcriptional regulation of promoter P3 and P4 in human HCC cells, Hep3B and HepG2. However, there is little information on the binding patterns of transcriptional factors in IGF-II promoter P3 in cirrhotic liver tissue and HCC tissues.

In the present study, in order to understand how

IGF-II gene expression is regulated in cirrhosis and HCC, we examined the binding pattern of transcriptional factors within promoter P3 that is predominantly used in fetal and tumor stage (12, 13) using nuclear extracts from human liver cirrhosis and HCC tissues, which were considered as the steps of hepatocarcinogenesis, compared to normal liver and fetal liver tissue by EMSA and DNAse I footprinting assay and identified tissue-dependent factors in these human extracts.

MATERIALS AND METHODS

Plasmids and probes

For EMSA, DNA fragments were obtained as follows. An *ECoR* I-*Sal* I fragment [−291:+135] and fragments covering IGF-II promoter P3 [−1230:−291] from HUP3 vector (14) were prepared for promoter P3 analysis. All these fragments were digested with double restriction enzymes, dephosphorylated and end-labelled with [γ-^{32}P] ATP and T4 kinase.

For DNase I footprinting assay, HUP3 was digested with *ECoR* I, dephosphorylated and end-labelled with [γ-^{32}P] ATP and T4 kinase. Double end labelled DNA was digested with the same restriction enzyme used in EMSA, *Sal* I.

Cell culture

Two cell lines derived from human tumors, HepG2 and HeLa purchased from ATCC (American Cultured Type Collection, Rockville, Maryland), were cultured in modified Eagle's medium (MEM) and DMEM, respectively, supplemented with 10% fetal bovine serum with sodium pyruvate, L-glutamin and penicillin/streptomycin (Gibco BRL, Bethesda, Maryland) in a humidified atmosphere at 37℃ and 5% CO_2. The cells were grown to a density of 4×10^6 cells/ml and maintained by dilution to 2×10^5 cells/ml with fresh complete medium every 2 days. Confluent cells were scraped into 1 ml of ice-cold PBS with protease inhibitors (1 μg/μl pepstatin, 1 μg/μl benzamidine, 1 mM phenylmethylsulfonylfloride), pelleted and kept ready until use in a deep freezer. Cell viability was monitored by MTT [3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide (sigma)] assay as described (15).

Human tissues

Tissue samples were taken from liver tissues resected during surgery from 5 patients with liver cirrhosis and 7 patients with hepatocellular carcinoma. Both tumorous and cirrhotic background tissues were available in 3 of 7 resected HCC samples. These 3 samples were used in EMSA and footprinting assay. The patients with liver cirrhosis and/or HCC had a mean age of 57 years, ranging from 42 to 69 years. Their serological findings were seropositive for HBsAg and seronegative for anti-HCV (hepatitis C virus). For comparison, human fetal liver tissues from 24 week- and 19 week-old fetus and normal adult liver tissue were used.

Preparation of nuclear extracts from cell line

Nuclear extracts from HepG2 and HeLa cell lines were prepared according to Dignam et al. (16) with minor modification. Cells were harvested by centrifugation for 10 min at 2,000 rpm. Pelleted cells were washed with cold PBS, and resuspended in five packed cell pellet volume of buffer A (10 mM Tris, pH 7.6, 1.5 mM $MgCl_2$ 10 mM KCl), supplemented with 0.5 mM PMSF (phenylmethylsulfonyl chloride). After standing on ice for 10 min, cells were centrifuged at 2,000 rpm for 10 min, resuspended in two packed cell pellet volume of buffer A, and lysed by 10 strokes of Dounce homogenizer (B type pestle). Nuclei were pelleted at 10,000 rpm for 10 min and resuspended in three packed nuclei volume of buffer C (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 25% glycerol, 0.42 M NaCl, 0.2 mM EDTA) containing 0.5 mM DTT and 0.5 mM PMSF. Nuclear proteins were extracted by stirring at 4℃ for 30 min. After centrifugation at 15,000 *g* for 30 min, the supernatant was dialysed against buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA) with 0.5 mM DTT and 0.5 mM PMSF. The dialysate was clarified by centrifugation at 25,000 *g* for 60 min at 4℃, aliquoted and stored in a deep freezer. The concentration of protein was determined by the bichinconic acid assay system (Pierce, Rockford, IL)

Preparation of nuclear extracts from human tissues

Each batch of liver extracts were prepared from the resected human tissues as described by Hames et al. (17), with minor modification. Two to 20 g of liver samples from normal adult liver, fetal, liver cirrhosis and HCC tissues were separately homogenized with homogenization buffer (10 mM HEPES, pH 7.6, 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2.4 M sucrose, 1% low fat milk, 10 g/60 ml) with 0.5 mM DTT, 0.5 mM PMSF and 1% protease inhibitor using Potter-Elvejem homogenizer by four strokes, and centrifuged at 75,000 *g* for 60 min. With a spatula, the solid pellicle that was floating in the tube was aspirated off. After checking the nuclei under the microscope, the nuclei was

resuspended in nuclear lysis buffer (10 mM HEPES, pH 7.6, 0.1 M KCl, 0.1 mM EDTA, 10% glycerol, 3 mM $MgCl_2$, 5 ml/10 g of raw liver tissue) containing 1 mM DTT and 0.1 mM PMSF and homogenized for several strokes with a Dounce homogenizer fitted with an A pestle. 5 M NaCl was added to a final concentration of 0.4 M. The mixture was mixed gently and left on ice for 30 min with occasional shaking. After centrifugation at 90,000 *g* for 60 min, the supernatant was transferred to a new tube and dialyzed against 100 V of dialysis buffer (25 mM HEPES, pH 7.6, 0.1 mM EDTA, 40 mM KCl, 10% glycerol) with 1 mM DTT for at least 5 hrs. The dialysate was centrifuged for 2 min and clear supernatant was aliquoted and kept at -80 C. The concentration of protein was determined as described above.

Electromobility shift assay (EMSA)

Twenty μg of nuclear proteins were incubated with binding mix (10 mM Tris pH 7.5, 50 mM NaCl, 0.5 mM DTT, 5 mM $MgCl_2$, 5 mM EDTA, 0.05% nonidet P-40) in a final volume of 20 μl containing 20 μg/ml poly dIdC, 0.5 mg/ml bovine serum albumin for 15 min on ice and shifted to 25℃ for 20 min with 20,000-100,000 cpm of a radiolabelled probe. The products were resolved by electrophoresis through 5% native polyacrylamide gel in 0.5 X TBE (22.5 mM Tris-Borate, 0.5 mM EDTA). The gel was dried and exposed to X-ray film (18).

DNase I footprinting assay

Binding reaction of 50 μl (10 mM Tris pH 7.5, 50 mM NaCl, 0.5 mM DTT, 5 mM $MgCl_2$, 5 mM EDTA, 0.05 % nonidet P-40, 20 μg/ml poly dIdC, 0.5 mg/ml bovine serum albumin) containing 20 μg of nuclear extract with 20,000 cpm of a radiolabelled probe, were carried out for 20 min at room temperature after incubating on ice for 15 min. Fifty μl of a room temperature solution containing 5 mM $CaCl_2$ and 10 mM $MgCl_2$ was subsequently incubated for 1 min. DNase I (Promega, 3 U/ml) was then added for 1 min. The reaction was terminated by adding 140 μl of DNase I stop solution (192 mM sodium acetate, 32 mM EDTA, 0.14% SDS, 64 μg/ml yeast tRNA), extracted with phenol:chloroform (1:1), precipitated with ethanol and finally separated on a 6% sequencing gel (19).

For immunoprecipitation, polyclonal anti-rabbit Egr1, Egr2, Sp1, c-jun and c-fos was added to the binding assay buffer containing nuclear proteins at room temperature for 2hr before addition of radiolabelled probe. The antibodies used in this study were rabbit polyclonal IgG, all purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Western blot analysis

For detection of IGF-II in total lysates from human liver tissues including normal, fetal, cirrhosis, HCC tissues and two cell lines, two to 5 grams of liver tissue was homogenized in acid-ethanol solution (85% ethanol, 12 N HCl) (20) that frees IGF-II from IGF-II binding proteins and incubated overnight at 4℃ with minor shaking. The homogenate was centrifuged at 12,000 rpm for 15 min at 4℃. The supernatant was transfered to a new tube, lyophilized and dissolved in 80 mM Tris buffer. Thirty μg of total proteins on 15% SDS-polyacrylamide gel was electrophoresed according to Laemmli (21) at 250 V/30 mA at 4℃ in Tris-glycine buffer (25 mM tris pH 8.3, 250 mM glycine, 0.1% SDS). Proteins were transferred to NC membrane in transfer buffer (25 mM tris pH 8.3, 250 mM glycine, 0.1% SDS, 20% methanol). Membrane was blocked for 1 hr in TBS-T (10 mM Tris-Cl, pH 7.6, 150 mM NaCl, 0.1% Tween 20) containing 5% non fat milk, followed by incubation for 2 hr with 1 μg/ml of anti-IGF II in TBS-T. Horse raddish peroxidase conjugated secondary antibody was treated for 1 hr at room temperature after washing with TBS-T three times. Detection was via enhanced chemiluminescence according to manufacture's directions for 1 min and then exposure to X-ray film (Amersham, Life Science, England).

RESULTS

IGF-II in total proteins from human liver tissues

For the initial screening of IGF-II expression, Western blot analysis was performed for three samples which had both tumorous and cirrhotic background tissues. As shown in Fig. 1, IGF-II protein was detected at the molecular weight of 20.5 and of 22 kDa with weak intensity in normal (NL) and liver cirrhosis (LC), of which a small size of 20.5 kDa was dominant in LC tissue. However in fetal liver (FL) and in HCC tissue, a big size of 52 kDa was observed, although there was a different pattern in minor band, 22 kDa in FL and 20.5 kDa in HCC tissue. A big size of IGF-II was the major form both in FL and in HCC tissues.

The detection of IGF-II in total lysate from normal liver tissue was similar to that of LC lysate, of which the small size of 20.5 kDa was abundant in LC tissue. Although the big size, 52 kDa, was detected both in fetal and HCC lysates, there were different sizes of minor band, 22 kDa in FL and 20.5 kDa in HCC tissue.

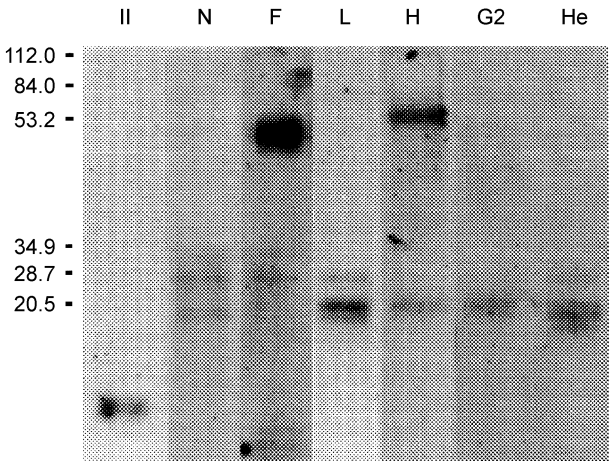

Fig. 1. Detection of IGF-II in total cell lysates from human liver tissues. 50 μg of total lysate was separated on 15% SDS-PAGE, transferred on NC membrane. The membrane was incubated with mouse monoclonal anti-rabbit IGF-II and developed with ECL. II: recombinant human IGF-II used as a positive control, N: normal liver, F: fetal liver, L: liver cirrhosis, H: hepatocellular carcinoma, G2: HepG2, hepatocellular carcinoma cell line, He: HeLa, human cervical cancer cell line.

The binding pattern of EMSA in promoter P3

In order to evaluate the difference in binding pattern of transcriptional factors in IGF-II promoter P3 [−291: +135], EMSA was performed with nuclear extracts prepared from human normal adult, fetal, LC and HCC tissues. For comparison, nuclear proteins from HepG2 cells that express endogeneous IGF-II, and HeLa cells which do not express endogeneous IGF-II were also used. Four fragments covering [−1230:+135] of promoter P3 were analysed by EMSA. Nuclear extracts from two cirrhotic tissues of three tissue samples which had both tumorous and cirrhotic background were tested. Both cirrhotic liver extracts showed the same retarded pattern, but with a little differences in intensity. Three individual HCC nuclear extracts from three samples with both tumorous and cirrhotic background also showed the same pattern in EMSA. Fig. 2 shows the different binding patterns and intensities of retarded complexes among FL, LC and HCC tissues, in which 5, 5 and 2 bands were obtained, respectively. Complex 1 was a common band found in FL, LC and HCC extracts with different intensity, showing that the intensity of complex 1 was strong in HCC and decreased in the order of LC to FL tissues. Complex 2 in FL and in LC tissue was seen with different binding intensities, strong in FL, but not seen in HCC. Complex 3 was strong in HCC, very weak in LC but not found in FL tissue. Whereas complex 4, 6 and 7 were observed only in FL tissue, Complex 9 and 10 were found in LC tissue only. Nuclear extracts from cell lines showed

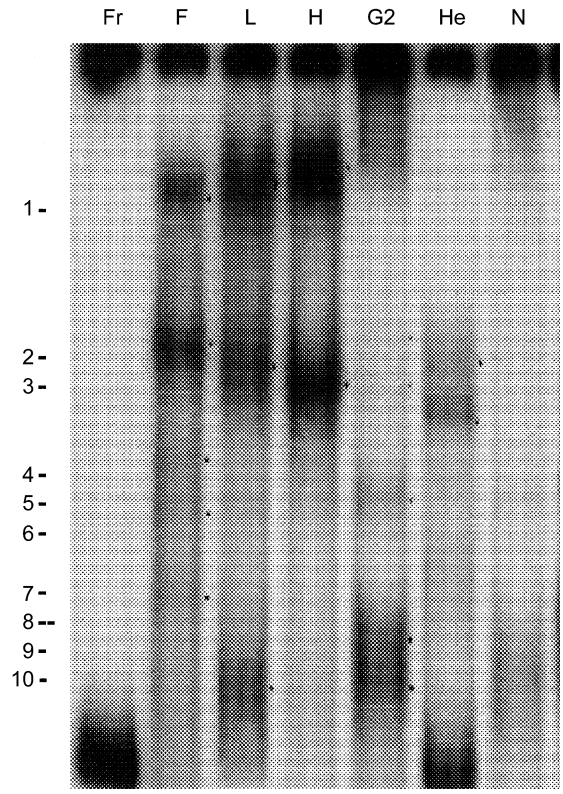

Fig. 2. Electromobility shift assay of human IGF-II promoter P3 [−291:+135] with nuclear extracts from human liver tissues. Twenty μg of crude nuclear extracts were added to reaction mix, followed by adding double end-labelled DNA probe. Reaction mixtures were electrophoresed on 5% non-denaturing polyacrylamide gel with 0.5X TBE. Fr: free probe, N: normal liver, F: fetal liver, L: liver cirrhosis, H: hepatocellular carcinoma, G2: HepG2, hepatocellular carcinoma cell line, He: HeLa, human cervical cancer cell line.

different binding patterns; complex 2 and 3 were observed in HeLa and complex 5, 8 and 9 were observed in HepG2. NL extract had only one band of complex 2.

The major bands were complex 1 and 2 in FL tissue with weak intensity, complex 1, 2 and 3 in LC tissue with moderate intensity, and complex 1 and 3 in HCC tissue with strong intensity, respectively. While in HepG2, complex 2 and 3 were major complexes with very weak intensity, and complex 8 and 9 were in HeLa with weak intensity. These results indicate that there are quantitative and qualitative differences in binding patterns of transcriptional factors with IGF-II promoter P3 according to the human liver tissue extracts used in the present study.

Footprinting analysis of promoter P3

Two cirrhosis nuclear extracts which showed the same retarded pattern in EMSA showed a little differences in

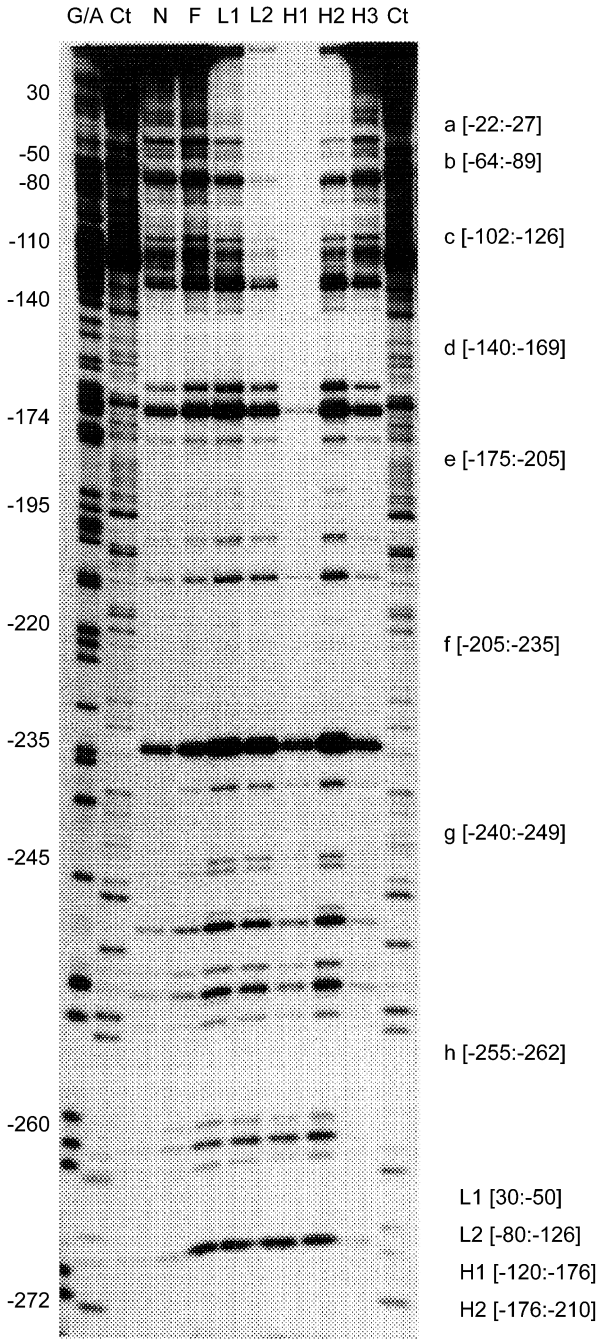

Fig. 3. DNAse I footprinting assay of human IGF-II P3 [-291: +135] with nuclear extracts from human liver tissues. The single end-labelled DNA probe was added to 50 μl of reaction mix containing 20 μg of crude nuclear extract. DNase I was treated for 1 min and separated on 6% denatured polyacryamide sequencing gel with 1X TBE. G/A is a Maxam-Gilbert sequencing reaction of adenines and guanines. Ct: without protein, N: normal liver, F: fetal liver, L1-2: liver cirrhosis, H1-3: hepatocellular carcinoma.

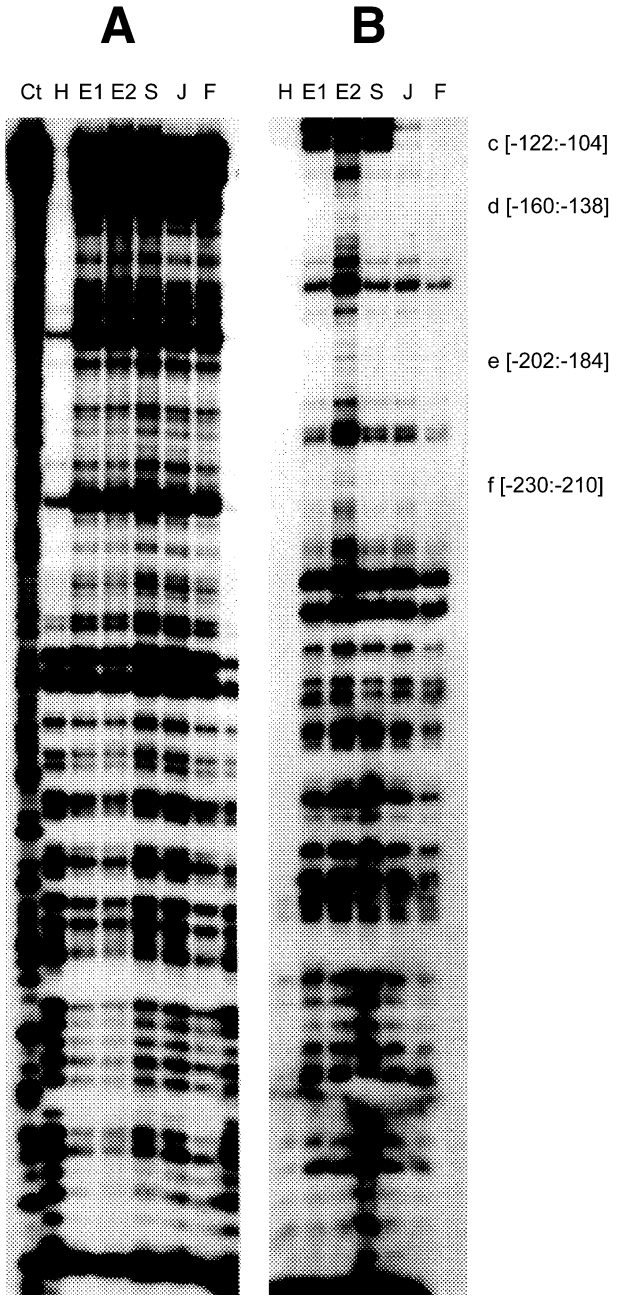

Fig. 4. Footprinting assay after immunoprecipitation of human IGF-II promoter P3. Nuclear proteins preincubated with specific polyclonal anti Egr1, Egr2, Sp1, c-jun and c-fos, were reacted with single end labelled DNA probe, digested with DNAse I and separated on sequencing gel with 1X TBE. (A) After centrifugation of immune complex, supernatant was assayed. (B) Preincubated nuclear mix was directly used in footprinting assay. E1: preincubated with polyclonal anti-Egr1, E2: with Egr2, Sp: with Sp1, J: treated with c-jun, F: with c-fos.

the a-c regions in footprinting, and three nuclear extracts from HCC also showed a little variations in the same regions. Eight common footprints, region from a to h, were observed in all lanes, human NL, FL, LC and HCC tissues, as demonstrated in Fig. 3. These footprints included TATA box (region a) and CCAAT box (region

b). The footprints of region c[−126:−102] and f [−205:−235] have the homology to Egr1, which was overlapped by the binding sequence of wt1 and Sp1. Region e [−205:−175] does not have any consensus sequences of known transcriptional factor(s). Region d [−169:−140] has a Sp1-like sequence. We could find footprints of region g and h which did not show any sequence homology to other TF sequences. However, the nuclear proteins from LC and HCC tissue showed different footprinting patterns; LC extract had specific L1 [−80:+30] which contains Egr and/or Wt1 binding sequence, and L2 [−126:−80] that is similar to region b plus c, but HCC tissue extract had not only two L-specific but also H-specific, H1 [−176:−120] and H2 [−210:−177].

Footprinting analysis after immunoprecipitation

For footprinting after immunoprecipitation, HCC extract indicated as H_1 in Fig. 3 was used. The fragment of human IGF-II P3 [−291:−98] which was single end labelled and the nuclear extracts from human HCC preincubated with specific antibodies to Egr1, Egr2, Sp1, c-jun and c-fos, were used in footprinting assay. After centrifugation of immune complex, all footprinting regions disappeared, but reaction with immune complex in binding reaction showed different results. Region c was protected in the presence of Egr1, Egr2 and Sp1 antibody, respectively, which has the consensus sequence of Egr1/wt1, and was still protected in the presence of c-jun and c-fos antibody, as shown in Fig. 4A and 4B. Although nuclear extract preincubated with Egr2 showed a slightly different pattern of footprint that was partially protected compared to nuclear extracts treated with other antibodies, a typical recover of footprinting was not observed in the region d [−160:−138], e [−202:−184] and f [−230:−210]. This indicates that, because antibodies cannot bind DNA binding domain of specific antigen, transcriptional factors, steric hinderance by modification of molecular structure forming immune complex might be involved in the disappearance of footprinting of region c. This result might be an indication that promoter P3 of human IGF-II might be transcriptionally regulated by Egr1, Egr2 and Sp1 directly and by c-jun and c-fos indirectly.

DISCUSSION

To investigate the molecular basis in the regulation of human IGF-II expression and to analyze the sequence and factors involved in transcriptional regulation of the major fetal promoter P3, we performed EMSA with nuclear extracts from human NL, FL, LC and HCC tissues. We could not find any difference in binding pattern and binding intensity using IGF-II P3 fragments covering [−1230:−291]. However, the proximal region [−291:+135] showed a clear different result. Complex 2 in FL and in LC tissue, and complex 3 in HCC was a dominant retarded band of IGF-II promoter P3, although complex 1 was retarded with three kinds of extract from FL, LC and HCC tissues, with different intensity, showing strong in HCC, moderate in LC and weak in FL.

Human tumor cell line, Hep3B that uses promoter P3 dominantly was studied by Schneid et al. (18). They found eight retarded complexes in region [−294:−71] and 8 retarded complexes in region [−154:+135] within IGF-II P3. Although the labelled probes overlapped each other, at least more than 8 complexes were found in IGF II P3 [−294:+135]. However, nuclear extract from HepG2 in our data showed 5 retarded bands in this region. This discrepancy between their data and ours could be explained by the different cell lines used in their study and in the present study, even though both are derived from human hepatoma tissues and by different fragments, two fragment in theirs and one fragment in ours. Five retarded bands observed with nuclear extract from HepG2, were not found with nuclear extract from HeLa used as a negative control, indicating that there is a cell-type specific binding pattern in IGF-II promoter P3. In addition, our data using tissue extracts showed there were clear retarded bands in each extract, 5 bands in FL, 5 in LC and 2 in HCC tissues, respectively. From EMSA, it could be concluded that human IGF-II promoter P3 might be regulated by cell-type specific and tissue-specific transcriptional factors in different human tissues.

To define the regulatory elements present in IGF-II promoter P3, we carried out footprinting assay using P3 fragment [−291:+135] with nuclear extracts from human liver tissues including NL, FL, LC and HCC. Eight common footprints were observed in all tissue extracts. Among the eight common footprints, region c [−126:−102] and region f [−205:−235] have Egr/wt1 binding sequence and Sp1 binding sequence. The nuclear extracts from LC showed L-specific, L1 [−80:+30] and L2 [−126:−80] in addition to common footprints, and those from HCC tissue showed HCC-specific, H1 [−176:−120] and H2 [−210:−177] as well as L-specific. IGF-II in FL tissue might be regulated by transcriptional factors which could protect the common footprinting region, but overexpression or amplification of IGF-II in LC may be controlled by L-specific sequence binding factors that have Egr1 as well as common footprinting factors. IGF-II gene in HCC tissue might be controlled by H-specific sequences as well as L-specific and common transcriptional factors. H-specific sequences include Egr1 binding sequence within L2-region, SP1 binding sequence within H1 region and sequence that does not have any homol-

ogy to known TF sequence within H2 region.

Raizis et al. (11) performed DNAse I footprinting assay using human IGF-II P3 [−291:+135] to analyze the structure of promoter P3 and showed that there are 9 footprintings including TATA, CCAAT, Sp1-like sequence and Egr1. Their result is in agreement with ours on common footprints, although they used HepG2 cell line, instead of human tissue extracts as used in the present study. However, it was also reported by other workers (8) that this region of IGF-II P3 has 5 footprintings including TATA, CCAAT, Sp1 site and two unknown factors. The difference between their findings and ours might be caused by the different display of footprinting data. Because they showed only a fragment [−200:+1], we could not observe the regions covering [−295:−200] and [+1:+135]. However, the nuclear extract from cell lines did not show any L-specific and H-specific footprints.

To evaluate which kinds of transcriptional factors that regulate IGF-II gene expression can bind to promoter P3 [−291:+135], DNase I footprinting assay was performed using human nuclear extracts with or without antibody, specific to TFs, from NL, FL, LC and HCC tissues. Whereas all footprints in promoter P3 [−295:−98] disappeared using supernatant of nuclear extract after centrifugation of immune complex containing Egr1, Egr2, Sp1, c-jun or c-fos antibody, the footprints were alive when nuclear extracts containing immune complex were used in the binding reaction. It is conceivable that immune complex-containing extracts showed similar footprinting as did crude extracts only, because each antibody does not bind to DNA binding domain of transcriptional factors. No footprints with supernatant after centrifugation to remove immune complex could be explained by the fact that all antigen makes immune complex with antibody and could be removed by centrifugation, which results in the disappearance of footprints. In spite of steric hindrance by forming immune complex, antibody to Egr1, Egr1 and Sp1 could recover footprints partially, indicating these TFs including Egr1, Egr2 and SP1 might be bound to these regions but c-Jun and c-Fos does not bind to this promoter P3 element directly.

In conclusion, the expression of IGF-II gene in LC tissues might be regulated by L-region binding transcriptional factor like Egr1 and common transcriptional factors such as TATA, CCAAT binding proteins used in FL tissue. In HCC tissue, overexpression of IGF-II could be mediated by H-region binding factors including SP1, L-region binding factors and unknown factors. It is, therefore, conceivable that identification of the pathophysiological conditions that enhance the expression of stage specific TFs, Egr1 in LC and Sp1 in HCC and evaluation of the function of these proteins could aid on the better understanding of IGF-II overexpression in LC and HCC. In this study, however, it should be stressed the following points. Firstly, although we had considerable practical problem to protect the stability of mRNA from operated human materials which takes several hours for complete surgical resection and eventual arrival to the laboratory, several TFs observed in the cirrhotic and HCC tissues should be matched P3 promoter-related mRNA transcripts by Nothern blot. This study may provide more information on the role of TFs identified in this work. Secondly, much more study is still required to identify the transcriptional factors that bind to the unknown TF binding element in H2 region and to determine how these TFs could function and which factors play an essential role in IGF-II gene expression.

REFERENCES

1. Worth Health Organization. *Advances in viral hepatitis. Report of the WHO Expert Committee on Viral Hepatitis. WHO Tech Rep Ser 1977; 602: 7-47.*
2. Park BC, Huh MH, Seo JH. *Differential expression of transforming growth factor α and insulin-like growth factor II in chronic hepatitis B, cirrhosis and hepatocellular carcinoma. J Hepatol 1995; 22: 286-94.*
3. Sussenbach JS. *Progress Growth factor Res 1990; 1: 33-48.*
4. Ward A, Ellis CJ. *In Schofield PN (ed). The insulin like growth factors-structure and biological function. Oxford University Press, Oxford 1992: 46-79.*
5. Volepert O, Jackon D, Bouck N, Linzer DIH. *The insulin like growth factor II/mannose 6-phosphate receptor is required for proliferation-induced angiogenesis. Endocrinology 1996; 137: 3871-6.*
6. Sussenbach JS, Holthuizen P, Steenbergh PH. *Structure and expression of the human insulin like growth factor genes. Growth Regulation 1992; 2: 1-9.*
7. Hothuizen PE, Rodenberg RJT, Van Dijk MA, Koonen-Reemst AMCB, Charnay P, Sussenbach JS. *Transcriptional regulation of the human IGF-II gene. Ann NY Acad Sci 1993; 684; 214-7.*
8. van Dijk FMA, Van Schaik HJ, Hothuizen PE, Sussenbach JS. *Initial charaterization of four promoters of the human insulin like growth factor II gene. Mol Cell Endocrinol 1991; 81: 81-94.*
9. van Dijk MA, Rodenburg RJT, Holthuizen PE, Sussenbach JS. *The liver-specific promoter of the human insulin like growth factor II gene is activated by CCAAT/enhancer binding protein (C/EBP). Nucleic Acids Res 1992; 12: 3099-104.*
10. van Dijk MA, Holthuizen PE, Sussenbach JS. *Elements required for activation of the major promoter of the human insulin like growth factor II gene. Mol Cell Endocrinol 1992; 88: 175-85.*
11. Raize AM, Eccles MR, Reeve AE. *Structural analysis of the human insulin like growth factor II P3 promoter. Biochem J*

1993; 289: 133-9.

12. Gray A, Tam AW, Dull TJ, Hayflick J, Pintar J, Cavenee WK, Koufos A, Ullrich A. *Tissue specific and developmentally regulated transcription of insulin like growth factor 2 gene. DNA 1987; 6: 283-95.*
13. Cariani E, Lasserre C, Kemeny F, Franco D, Brechot C. *Expression of insulin like growth factor II, alpha-fetoprotein and hepatitis B virus transcripts in human primary liver cancer. Hepatology 1991; 13: 644-9.*
14. Hothuizen PE, Cleutjens CBJM, Veenstra GTC, van der Lee FM, Koonen-reemst AMCB, Sussenbach JS. *Differential expression of the human, mouse and rat IGF-II gene. Regul Pept 1993; 48: 77-89.*
15. Tada H, Shino O, Kuroshima K, Koyama M, Tsukamoto M. *An improved calorimetric assay for interleukin-2. Eur J Immunol 1986; 93: 157-65.*
16. Dignam J, Lebowitz RM, Rofer RG. *Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 1983; 11: 1475-89.*
17. Hames BD, Higgins SJ. *Gene transcription. A practical approach. IRL press, Oxford, Oxford University, 1993: 129-34.*
18. Schneid H, Hothuizen PE, Sussenbach JS. *Differential promoter activation in two human insulin like growth factor II producing tumor cell lines. Endocrinology 1993; 132: 1145-50.*
19. Briggs MR, Kadonaka, JT, Bell SP, Tijan R. *Purification and biochemical charaterization of the promoter-specific transcription factor, Sp1. Science 1986; 234: 47-52.*
20. Romanus JA, Yang YWH, Adams SO, Sofair AN, Tseng LYH, Nissley SP, Rechler MM. *Synthesis of insulin like growth factor II in fetal rat tissues: Translation of IGF-II ribonucleic acid and processing of pre-pro-IGF-II. Endocrinology 1988; 122: 709-16.*