Structure and Expression of the Rat c-*jun* Messenger RNA: Tissue Distribution and Increase during Chemical Hepatocarcinogenesis

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Structure and Expression of the Rat c-*jun* Messenger RNA: Tissue Distribution and Increase during Chemical Hepatocarcinogenesis[1]

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ABSTRACT

c-*jun* is the cellular homologue of the recently isolated nuclear oncogene v-*jun*. This protooncogene encodes the cellular transcription factor AP-1. We have isolated the complementary DNA clone of rat c-*jun* mRNA. The rat c-*jun* complementary DNA clone encodes 334 amino acid residues, the sequence of which shows about 98, 96, and 81% homologies with mouse, human, and chicken c-*jun* products, respectively. The c-*jun* mRNA is highly expressed in the lung and slightly expressed in the brain. The amount of mRNA is even smaller in the liver and is not detected in the spleen. We have also determined c-*jun* expression during chemical hepatocarcinogenesis and demonstrated increased expression of mRNA in the precancerous lesion, hyperplastic nodules, as well as in the primary hepatocellular carcinomas.

INTRODUCTION

We have been studying the expression mechanisms of the rat GST-P[3] gene during hepatocarcinogenesis. GST-P is expressed specifically from the early stages of chemical hepatocarcinogenesis to hepatocellular carcinomas in the rat (1, 2). Because of the extremely high coincidence between GST-P expression and (pre)neoplastic transformation of rat hepatocyte, this enzyme has been used widely as an early tumor marker in this system (3–9). In an attempt to study the regulatory elements of the GST-P gene, we have found two copies of TRE-like sequences in the 5′-upstream region of this gene (10, 11). We have also shown that GST-P gene is activated by TPA in a certain cell line (11). Recent reports indicate that transcription factor AP-1, encoded by c-*jun* gene, binds to TRE sequence and stimulates transcription of the gene containing TRE (12–16). c-*jun* is transiently activated during G_0-G_1 transition and this expression pattern is quite similar to that of the c-*fos* and c-*myc*, which are called the immediate early genes (17, 18). These reports suggest that the c-*jun* product is closely related to the progression of the cell cycle through G_1. More interestingly, another nuclear oncogene c-*fos* product specifically associates with c-*jun* product and amplifies the transactivator function against TRE-containing genes (19–21). For further studies of regulation mechanisms of GST-P gene, particularly in terms of the relationship between GST-P gene and the c-*jun*, we have isolated the cDNA clones of the rat c-*jun* mRNA. Here, we report the primary structure of rat c-*jun* mRNA and the deduced amino acid sequence. We also show its tissue distribution and changes in expression during chemical hepatocarcinogenesis.

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[3] The abbreviations used are: GST-P, glutathione transferase P; cDNA, complementary DNA; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TRE, TPA-responsive element; poly(A), polyadenylate; DEN, diethylnitrosamine.

MATERIALS AND METHODS

cDNA Cloning and Sequencing. A cDNA library against rat liver poly(A)-containing RNA was constructed by using a λgt10 cDNA cloning kit (Amersham, United Kingdom) according to the protocol recommended by the supplier. The cDNA library was screened with synthetic oligodeoxynucleotide probes complementary to the human c-*jun* cDNA. Two of the 36-mer oligodeoxynucleotides complementary to the nucleotide positions from 469 to 504 (NH_2-terminal probe) and from 1286 to 1321 (COOH-terminal probe) of the human c-*jun* cDNA clone ch-J1 and ch-J2 isolated by Angel *et al.* (16) were synthesized and used for the screening. The synthetic probes were hybridized to a phage cDNA library immobilized on the filters in 1 M NaCl-20 mM Tris-HCl pH 8.0-1 mM EDTA-0.1% SDS, 10 × Denhardt's solution at 50°C and washed in 2 × SSC-0.1% SDS at 50°C. The isolated cDNA clones pRJ51 and pRJ101 were sequenced by the dideoxy method (22).

Chemical Hepatocarcinogenesis. The model of Solt and Farber (23) was used for the induction of hyperplastic nodules in rat liver. Briefly, 200 mg/kg body weight of DEN were injected i.p. into rats and the 0.02% *N*-2-acetylaminofluorene in standard diet was given for 2 weeks thereafter. Partial hepatectomy was done 3 weeks after DEN injection. Hepatocellular carcinomas were induced either by a diet containing 0.03% AAF or by 0.01% DEN in drinking water (1).

Southern Blot Analysis. Restriction endonuclease-digested rat liver DNA was separated by a 0.8% agarose gel and hybridized at 65°C for 18 h with ^{32}P-labeled pRJ101 DNA in a solution containing 1.0 M NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 × Denhardt's solution, and 0.1% SDS. Filters were washed for 30 min at 60°C in 0.2 × SSC-0.1% SDS. For washing under less stringent conditions, 1 × SSC or 5 × SSC were used instead of 0.2 × SSC.

Northern Blot Analysis. Total or cytoplasmic RNAs were isolated from rat tissues and from several cell lines. RNAs denatured by formaldehyde and formamide were separated on agarose gels containing 0.6 M formaldehyde and blotted on nylon membrane filters (24). Hybridization was carried out in 50% formamide-1% SDS-20 mM Tris-HCl-1 mM EDTA-0.9 M NaCl-10% dextran sulfate-5 × Denhardt's solution at 42°C for 18 h. Filters were washed in 0.4 × SSC-0.5% SDS at 60°C.

RESULTS

cDNA Cloning and Primary Structure of Rat c-*jun* mRNA. A cDNA library constructed from rat liver poly(A)-containing RNA was screened as described in "Materials and Methods." Six plaques of 2×10^5 plaques were positively hybridized with both COOH-terminal and NH_2-terminal probes. Two of the clones contained a 2.0-kilobase insert and four had a 2.6-kilobase insert. One of the clones containing a 2-kilobase insert (pRJ101) and another containing a 2.6-kilobase insert (pRJ51) were chosen for sequencing. In the pRJ101 clone, 553 nucleotides of 3′-terminal portion were missing as compared to the pRJ51. The nucleotide and deduced amino acid sequences are shown in Fig. 1*A* and the restriction maps in Fig. 1*B*. The pRJ51 clone containing 2579 nucleotides has a 1002-nucleotide open reading frame that encodes 334 amino acids. The calculated molecular weight of the protein encoded by pRJ51 is 36,000.35. The deduced amino acid sequence of pRJ51 is highly homologous to those of human, mouse, and chicken c-*jun* products. Comparison of the c-*jun* amino acid sequences of

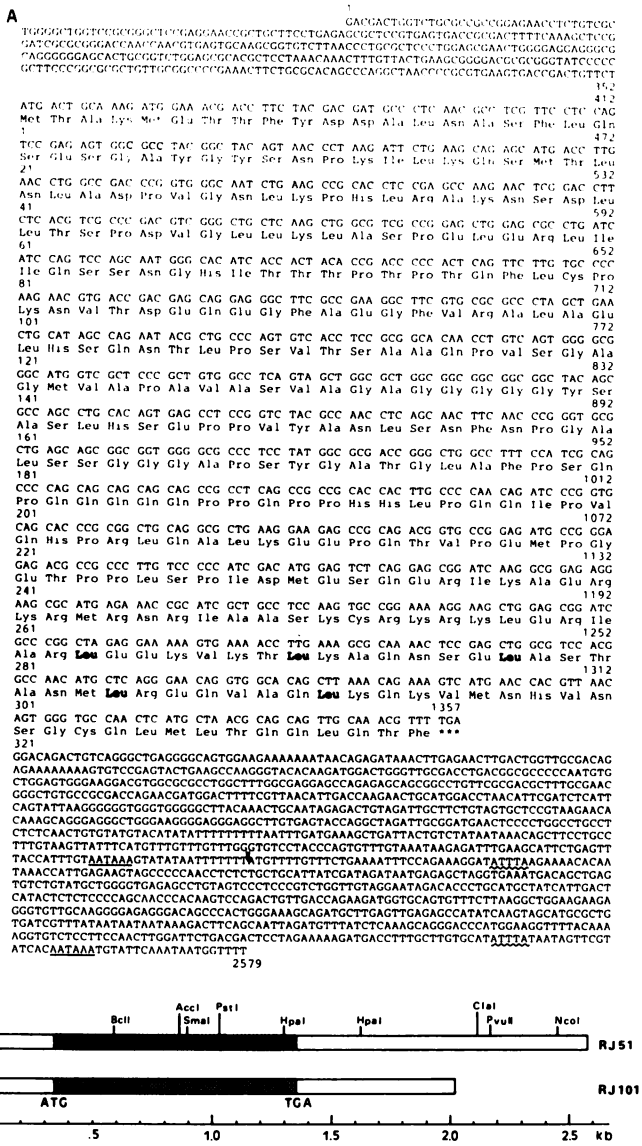

Fig. 1. Nucleotide and predicted amino acid sequence of the rat c-*jun* cDNA. *A*, nucleotide and deduced amino acid sequence of pRJ51. *Right*, nucleotide positions; *left*, amino acid positions; *, termination codon. Two poly(A) addition signals are shown by *underbars*. The putative destability sequences, ATTTA, are indicated by *waved underbars*. Five leucine residues potentially forming a "leucine zipper" structure are *shadowed*. *Arrowhead*, the poly(A) attaching site of the shorter cDNA clone, pRJ101. *B*, restriction map of the rat c-*jun* cDNA clones, pRJ51 and pRJ101. *Shadowed areas*, the amino acid coding regions. *kb*, kilobases.

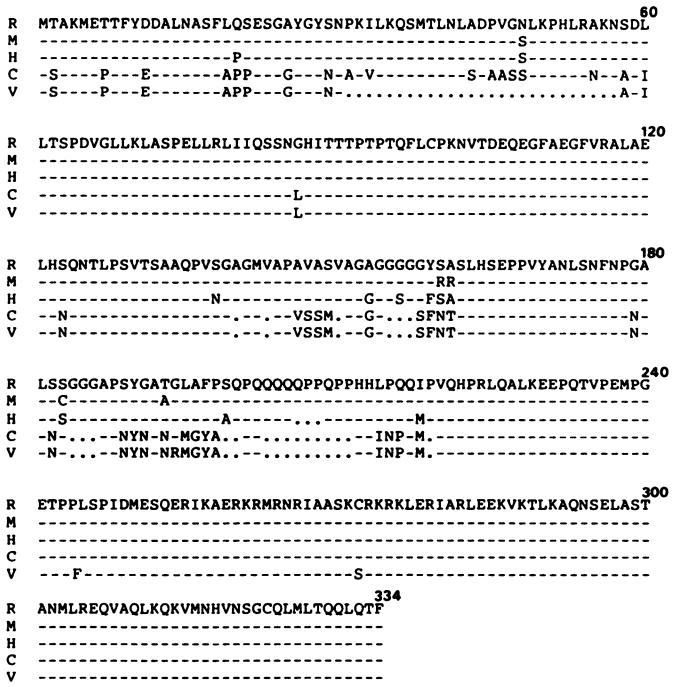

Fig. 2. Comparison of amino acid sequences of the c-*jun* proteins. The rat (*R*), mouse (*M*), human (*H*), chicken (*C*), and viral (*V*) *jun* products are compared using the computer program, GENETYX (Software Development, Ltd., Japan). — and ·, amino acids identical to and deleted from the rat c-*jun* protein, respectively.

these four species is shown in Fig. 2. The overall homologies of the rat c-*jun* amino acid sequence with human (16), mouse (17, 18), and chicken (25) are 96, 98 and 81%, respectively. The NH_2-terminal 60-amino acid sequences are highly conserved in mammalian species but considerably diverged from those of chicken. This region seems to be very important for the oncogenicity of the *jun* gene, as discussed by Nishimura and Vogt (25), because a 27-amino acid deletion in the v-*jun* sequence, which is the major difference between v- and c-*jun* sequence, is located in this region (Fig. 2). The next 62 amino acids (61st–122nd) and the COOH-terminal 116 amino acids (119th–334th) are almost completely identical among these species and viral *jun*. These results suggest that about 100 amino acids of the COOH terminus of the c-*jun* products are extremely important for the function of this protein. Landschulz *et al.* (26) have suggested that this domain may form a "leucine zipper structure" playing an important role for protein-protein interaction as well as protein-DNA interaction. The heptad repeat of the leucine residues, which can form a leucine zipper, is located on 283rd to 311st amino acid from the NH_2 terminus (Fig. 1*A*). The middle 97 amino acids (122nd–219th) are relatively diverged, *i.e.*, homologies of the rat c-*jun* product with humans, mouse, and chicken are 88, 96, and 55%, respectively. This central region may be functionally less important as compared to the NH_2- and COOH-terminal regions.

In the 3′ noncoding region, there are two poly(A) addition signals located at 2005 and 2554. The short mRNA which used the upstream poly(A) signal corresponds to the pRJ101 clone. The sequence related to instability of mRNA, ATTTA (27), is located at 2059 and 2533. No such sequence is found in shorter mRNAs (corresponding to pRJ101) but there are some related sequences such as GTTTA and T clusters.

Southern Blot Analysis of the c-*jun* Gene. Southern blot analysis of the rat genomic DNA using the rat c-*jun* cDNA as a probe detected a single band in *Eco*RI, *Bam*HI, and *Hind*III digests (Fig. 3). The *Eco*RI digest shows a band of about 2.5 kilobases which is very close to the size of the cDNA clone pRJ51 and mRNA (Fig. 4). Since no *Eco*RI site is present in the cDNA clone (except for attached linker sequences on both ends), it is unlikely that the c-*jun* gene has any long intron sequence. Indeed, no intron sequences are reported in human and chicken c-*jun* genes (25, 28). A single band was detected under highly stringent washing conditions, suggesting that the c-*jun* gene is unique in the rat genome. However, many extra bands were detected with lower stringency washing conditions. This suggests that there are many c-*jun*-related genes or sequences present in the rat genome. Some cDNA clones related to c-*jun* mRNA were also isolated (29).

Expression of c-*jun* in Rat Tissues. To determine the mRNA levels of c-*jun* in rat tissues, total RNAs were extracted from the liver, brain, spleen, testis, and lung and analyzed by Northern blot hybridization using pRJ51 as a probe. As shown in Fig. 4*A*, this gene is most actively expressed in the lung. A

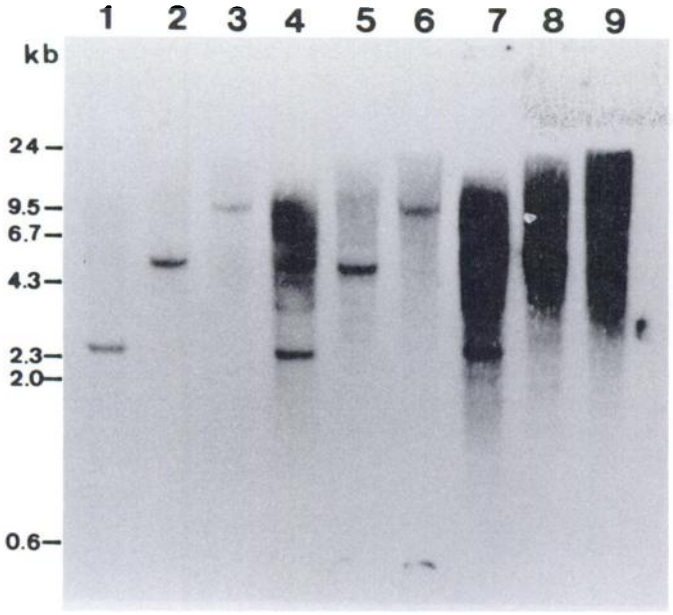

Fig. 3. Southern blot analyses of the rat c-*jun* gene. About 10 μg of rat genomic DNA were digested with either *Eco*RI (*Lanes 1, 4, and 7*), *Bam*HI (*lanes 2, 5, and 8*), or *Hind*III (*Lanes 3, 6, and 9*) and analyzed on a 0.8% agarose gel electrophoresis. Filters were washed under highly stringent conditions (*Lanes 1–3*), moderately stringent conditions (*Lanes 4–6*), and nonstringent conditions (*Lanes 6–9*), as described in "Materials and Methods." *kb*, kilobases.

small amount of mRNA was also detected in the brain. A very small amount of mRNA was detected by an extended exposure in the liver and testis but not in spleen. The amounts of c-*jun* mRNA in tissue culture cell lines and ascites hepatoma cell lines are shown in Fig. 4*B*. All rat hepatoma cell lines examined including AH130, Morris hepatoma 5123D, and dRLh84 highly expressed c-*jun* mRNA. HeLa cells contained relatively small amounts of c-*jun* mRNA. Undifferentiated F9 embryonal carcinoma cells did not have detectable amounts of c-*jun* mRNA. It has been reported that there was no AP-1 protein (c-*jun* product) in the undifferentiated F9 cell extract as determined by gel mobility shift assay and by foot printing analysis (30). The nucleotide sequence of rat c-*jun* cDNA is about 90% homologous with that of the human c-*jun* cDNA (amino acid coding region) and there are 90-nucleotide and 60-nucleotide stretches that are completely identical with each other. Therefore, it is inferred that the human c-*jun* mRNA was detected almost at the same efficiency as rat mRNA under the conditions used in these experiments. It should be noted that the experiments shown in Fig. 4 were done separately with different specific activity probes, different amounts of RNA applied, and different times of exposure to X-ray film, so that they are not directly comparable. From the results of other experiments, the hepatoma cell line dRLh84 cells appear to contain 2 or 3 times more c-*jun* mRNA than lung tissue.

Expression of c-*jun* mRNA during Chemical Hepatocarcinogenesis. Since all the hepatoma cell lines thus far studied highly expressed the c-*jun* mRNA, we now determined the expression of c-*jun* mRNA in early stages of the chemical hepatocarcinogenesis. According to the protocol of Solt and Farber, preneoplastic lesion hyperplastic nodules appeared 4–5 weeks after DEN administration. The RNAs from 4 and 6 weeks after DEN treatment were isolated as described in "Materials and Methods." The RNAs from primary hepatocellular carcinomas induced by *N*-2-acetylaminofluorene or DEN were also isolated. Fig. 5 shows Northern blot analysis of these RNAs hybridized with a *jun* probe. For controls of RNA quantitation and transfer, we have rehybridized the same filter with β-actin probe. The intensities of β-actin mRNA bands were almost similar in normal liver and hyperplastic nodule-bearing liver. Some of the primary hepatomas and the hepatoma cell line dRLh84, however, show significantly strong bands, although almost the same amounts of RNAs were applied. This may be because the β-actin gene contains serum-responsive element and rapidly growing hepatoma cells express higher amounts of β-actin mRNA than normal cells (31). At the 4th or 6th week after chemical carcinogen administration, c-*jun* mRNA in the treated liver was increased substantially as compared with normal liver. All of the five independently induced primary hepatocellular carcinomas expressed relatively high amounts of c-*jun* mRNA. In the hyperplastic nodules-bearing liver, however, nodule foci occupied approximately 15 and 60% of the whole liver mass at 4 and 6 weeks after carcinogen treatment (32), respectively.

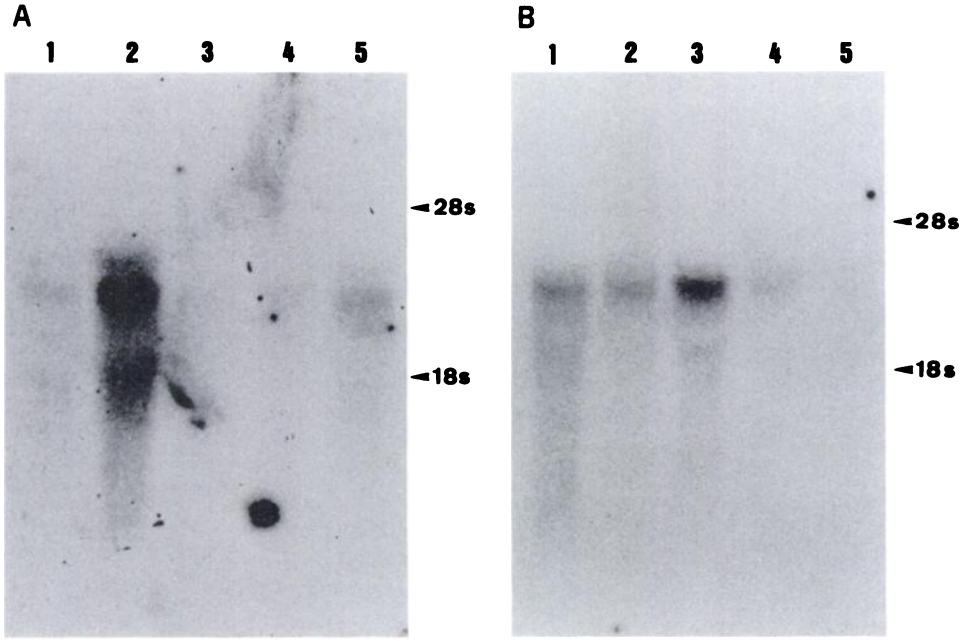

Fig. 4. Northern blot analyses of the c-*jun* mRNA. *A*, tissue distribution of the c-*jun* mRNA in the rat. About 50 μg of each RNA extracted from liver (*Lane 1*), lung (*Lane 2*), spleen (*Lane 3*), testis (*Lane 4*), and brain (*Lane 5*) were analyzed on denaturing agarose gels as described in "Materials and Methods." *B*, c-*jun* mRNA expression in the cultured cells and ascites hepatoma cells. Approximately 20 μg of each RNA extracted from rat ascites hepatoma AH130 (*Lane 1*), Morris hepatoma 5123D (*Lane 2*), a tissue culture hepatoma cell line dRLh84 (*Lane 3*), HeLa cells (*Lane 4*), or a human undifferentiated embryonal carcinoma cell line F9 (*Lane 5*) was analyzed.

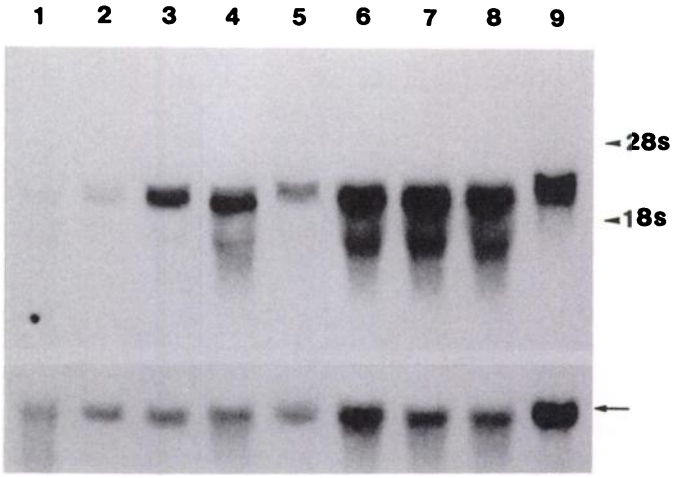

Fig. 5. c-*jun* expression during chemical hepatocarcinogenesis in the rat. The RNAs were extracted from the liver treated by the protocol of Solt and Farber (23) and hybridized with pRJ101 DNA. The filter was washed and rehybridized with β-actin cDNA for quantitation of transferred RNA. *Lane 1*, normal liver, *Lanes 2* and *3*, 4 and 6 weeks after carcinogen treatment, respectively. *Lanes 4–8*, hepatocellular carcinomas, independently induced by *N*-2-acetylaminofluorene (*Lanes 4*, *5*, and *6*) and DEN (*Lanes 7* and *8*). *Lane 9*, dRLh84 cells. Twenty μg of each RNA were analyzed. *Arrowheads*, 18S and 28S rRNA positions; *arrow*, β-actin mRNA.

Therefore, one can assume that the c-*jun* mRNA content of the hyperplastic nodule is comparable to that of the primary hepatocellular carcinoma cells. The highest amount of c-*jun* mRNA was found in the cultured hepatoma cell line, dRLh84.

DISCUSSION

Cloning of the Rat c-*jun* cDNA. We have cloned and sequenced rat c-*jun* cDNA. The transactivator function of cloned cDNA was confirmed by cotransfection experiments as follows. The cDNA insert of pRJ101 was joined to an expression vector containing human β-actin gene promoter and enhancer complex (33). This expression plasmid DNA and an indicator plasmid containing bacterial chloramphenicol acetyltransferase gene hooked to the human collagenase gene with a TRE sequence were cotransfected into the undifferentiated F9 cells. The chloramphenicol acetyltransferase activities of the transfected cells were demonstrated only after introduction of our c-*jun* expression plasmid DNA (data not shown).

Comparison of the amino acid sequences between rat, mouse, human, and chicken indicates a very high conservation of this protein. Especially 100 amino acids of COOH-terminal domain is almost identical among these species. The structural and functional significance of this domain has already been discussed in previous reports (34, 35).

Distribution of c-*jun* mRNA. Among the rat tissues studied, the lung most actively expressed the c-*jun* although what types of lung cells are really active in c-*jun* expression remain to be seen. The tissue distribution of the c-*jun* mRNA parallels fairly well that of the GST-P mRNA (36); *e.g.*, the lung expresses the highest amount of both c-*jun* and GST-P mRNA whereas normal tissue and precancerous liver, primary hepatocellular carcinoma, and some hepatoma cell lines express very high amounts of both mRNAs. The GST-P gene has two TRE-like sequences in its 5′-flanking region and is stimulated by TPA in a certain cell line (11). Gel mobility shift assay and footprint analysis suggested that an AP-1-like factor bound with the TRE-like sequence of GST-P gene.[4] These findings, together with the distribution of the GST-P mRNA and c-*jun* mRNA, suggest that the GST-P gene is regulated in major part by transcription factor AP-1 (c-*jun* product). On the other hand, the amount of c-*jun* mRNA or AP-1 protein alone cannot always explain the GST-P gene expression. For instance, an ascites hepatoma, AH130, expresses c-*jun* mRNA as much as a Morris hepatoma 5123D does. However, the former expresses almost no GST-P mRNA (36), while the latter expresses extraordinary amounts of it. Previously, we found that the GST-P gene having TRE-like sequence in its enhancer was highly expressed in the hyperplastic nodules as well as in the hepatocellular carcinomas. We now found that c-*jun* was also elevated in the early stages of liver carcinomas. To what extent, however, the c-*jun* expression during hepatocarcinogenesis is involved in the dramatic activation of GST-P gene remains to be determined.

In any event, the c-*jun* cDNA clone described here will facilitate the elucidation of the mechanism by which specific expression of the genes occurs that are related to neoplastic transformation and cell growth.

[4] Unpublished results.

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