JOURNAL OF VIROLOGY, Aug. 1996, p. 4978–4985 Vol. 70, No. 8
0022-538X/96/$04.00+0

Hepatitis B Virus HBx Protein Induces Transcription Factor AP-1 by Activation of Extracellular Signal-Regulated and c-Jun N-Terminal Mitogen-Activated Protein Kinases

JACQUELINE BENN, FEI SU, MARGHERITA DORIA,† AND ROBERT J. SCHNEIDER*

Department of Biochemistry and Kaplan Cancer Center, New York University Medical Center, New York, New York 10016

Received 16 January 1996/Accepted 22 April 1996

The HBx protein of hepatitis B virus is a dual-specificity activator of transcription, stimulating signal transduction pathways in the cytoplasm and transcription factors in the nucleus, when expressed in cell lines in culture. In the cytoplasm, HBx was shown to stimulate the Ras–Raf–mitogen-activated protein kinase (MAP kinase) cascade, which is essential for activation of transcription factor AP-1. Here we show that HBx protein stimulates two independently regulated members of the MAP kinase family when expressed transiently in cells. HBx protein stimulates the extracellular signal-regulated kinases (ERKs) and the c-Jun N-terminal kinases (JNKs). HBx activation of ERKs and JNKs leads to induction and activation of AP-1 DNA binding activity involving transient de novo synthesis of c-Fos protein and prolonged synthesis of c-Jun, mediated by N-terminal phosphorylation of c-Jun carried out by HBx-activated JNK. New c-Jun synthesis was blocked by coexpression with a dominant-negative MAP kinase kinase (MEK kinase, MEKK-1), confirming that HBx stimulates the prolonged synthesis of c-Jun by activating JNK signalling pathways. Activation of the c-*fos* gene was blocked by coexpression with a Raf-C4 catalytic mutant, confirming that HBx induces c-Fos by acting on Ras-Raf linked pathways. HBx activation of ERK and JNK pathways resulted in prolonged accumulation of AP-1–c-Jun dimer complexes. HBx activation of JNK and sustained activation of c-Jun, should they occur in the context of hepatitis B virus infection, might play a role in viral transformation and pathogenesis.

The human hepatitis B virus (HBV) X gene (HBx) synthesizes a 154-amino-acid (17-kDa) transcriptional transactivator (64, 65, 75) which has generally been viewed as a potential cofactor in viral carcinogenesis. HBx has proved to be an enigmatic polypeptide, possessing few structural features that indicate a mechanism of transcriptional activation. It does not bind DNA directly and contains no distinguishable homologies to families of other transcription regulatory proteins. A variety of transcription elements which are activated by HBx have been identified. Activation has been localized to binding sites for specific transcription factors such as NF-κB (36, 38, 40, 42, 55, 56, 64, 76), AP-2 (54), AP-1 (7, 8, 31, 49, 54, 63), and possibly c/EBP (26, 40, 66). HBx was also shown to strongly activate transcription by RNA polymerase III (6), which has recently been shown to occur by HBx activation of TFIIIB and increased levels of TATA-binding protein (69).

The mechanisms for transcriptional activation by HBx are becoming clearer. A number of studies have suggested that HBx might stimulate transcription at the promoter by enhancing the binding or activity of transcription factors and components of the transcription apparatus (28, 39, 54, 66). In vitro, HBx can bind the RPB5 subunit of RNA polymerase (16), TATA-binding protein (52), and ATF/CREB (39, 72), in the latter case altering its binding specificity. Other studies have suggested that HBx activates cytoplasmic signal transduction cascades (19, 34, 38, 49). Activation of signalling cascades was established when it was demonstrated that HBx activates Ras in vivo, leading to activation of Raf and mitogen-activated protein (MAP) kinases (7). Activation of the Ras-Raf-MAP kinase cascade was shown to be essential for HBx activation of AP-1 (7, 50, 60). At least a partial explanation may have recently been provided for the variety of conflicting activities reported for HBx. HBx was found to be distributed largely in the cytoplasm and to some extent in the nucleus in transfected cells (25). Cytoplasmic HBx was shown to activate the Ras-Raf-MAP kinase cascade, leading to induction of AP-1 and NF-κB, whereas nuclear HBx was found to stimulate HBV enhancer I by 5- to 10-fold.

Activation of Ras by different stimuli induces AP-1 by acting on several distinct protein kinases. Therefore, by investigating the Ras-dependent mechanism by which HBx transduces activation of AP-1, we can begin to establish the molecular basis for HBx activity. AP-1 is a dimeric complex of Fos and Jun leucine zipper proteins. Fos-Jun heterodimers are more stable and bind DNA more strongly than c-Jun homodimers (9, 11). The Fos and Jun proteins that comprise AP-1 are generally newly synthesized, although the activity of existing c-Jun protein is also regulated posttranslationally by phosphorylation (9, 12). In some studies, Ras activation has been linked to increased phosphorylation of N-terminal c-Jun sites that stimulate new c-Jun synthesis (9, 59). Phosphorylation of N-terminal sites is essential for autostimulation of new c-Jun synthesis (4, 5) and for transformation by c-Jun and oncogenic Ras (1, 59, 70).

Two groups of MAP kinases control the activation of AP-1. One group, the extracellular signal-regulated kinases (ERKs), were shown to be important Ras-dependent activators of the c-*fos* gene but are not directly involved in the control of c-Jun (30, 70). A second group of MAP kinases, termed JNKs, phosphorylate the N terminus of c-Jun (21, 22, 32, 35, 46, 57, 71). JNKs are strongly activated by UV light and in some systems more weakly by Ras. Activation of ERKs and JNKs is respon-

* Corresponding author. Phone: (212) 263-6006. Fax: (212) 263-8166. Electronic mail address: schner01@mcrcr6.med.nyu.edu.

† Present address: Instituto Europeo di Oncologia sre, Milan, Italy.

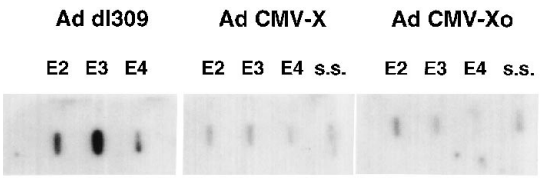

FIG. 1. Effect of HBx on transcriptional activation of Ad early region promoters E2, E3, and E4, determined by nuclear transcription runoff analysis. HepG2 cells were infected with 25 PFU of wild-type Ad *dl*309, Ad CMV-X, or Ad CMV-Xo per cell, nuclei were isolated and used for runoff analysis in the presence of [α-^{32}P]UTP, RNAs were extracted, and equal amounts (10^6 cpm per sample) were hybridized to slot blot filters containing an excess (500 ng per slot) of single-stranded probe specific for regions E2, E3, and E4 or denatured salmon sperm DNA (s.s. control). Ad CMV-X expresses the HBx gene under the control of the CMV promoter in a genetically silent, replication-defective Ad vector that lacks region E1. In Ad CMV-Xo, the HBx mRNA is deleted of all AUG codons. Autoradiograms were quantitated by scanning densitometry.

sive to nonoverlapping stimuli via different signalling cascades (23, 46, 61, 73); Ras activates the ERKs through a Raf pathway, while the JNKs are activated through a pathway controlled by MEKK-1 (45). Several studies demonstrated that the small GTP-binding protein Rac1 mediates strong activation of JNKs, whereas activation of Ras without stimulation of Rac1 leads to a moderate level of JNK activation (18, 44). JNK activation and N-terminal phosphorylation of c-Jun are believed to establish a positive autoregulatory loop that sustains c-Jun synthesis (4, 5).

In this study, we investigated the molecular mechanism for Ras-dependent activation of AP-1 by transiently expressed HBV HBx protein in Chang cells, a poorly differentiated hepatocyte cell line, and in HepG2 cells, a more highly differentiated hepatocyte cell line. We demonstrate that HBx induction of AP-1 involves activation of ERK and JNK MAP kinases, which together stimulate de novo synthesis of c-Fos and c-Jun polypeptides, respectively. Importantly, the composition of AP-1 complexes was found to change during the course of induction by HBx, from transient accumulation of c-Fos–c-Jun heterodimers to prolonged accumulation of c-Jun homodimers. The prolonged formation of c-Jun–AP-1 DNA binding complexes occurred posttranslationally by N-terminal phosphorylation of c-Jun, carried out by moderate activation of JNKs. Thus, HBx expressed transiently in hepatocyte cell lines (in the absence of HBV infection) is shown to act on two distinct pathways leading to activation of ERK and JNK MAP kinases.

MATERIALS AND METHODS

Cell culture. Cell lines used in this study were obtained from the American Type Culture Collection. Chang cells were propagated in modified Eagle's medium supplemented with 10% calf serum and 50 μg of gentamicin sulfate per ml. HepG2 cells were propagated in Ham's medium supplemented with 10% fetal calf serum and 50 μg of gentamicin sulfate per ml. Cells were made quiescent (serum starved) for 24 h in 0.5% calf serum prior to infection with adenovirus (Ad) vectors at 25 PFU per cell. After 1 h of infection, medium was made 2% in calf serum to facilitate protein synthesis and expression of HBx protein. Combined transfection-Ad infection experiments were carried out as described previously (7, 8). Briefly, subconfluent cell cultures were serum starved, then transfected with 10 μg of DNA per 10-cm-diameter plate with plasmids encoding catalytic mutants Raf-C4 (14) or MEKK-1 (45), and infected 5 h later with Ad vectors as described above.

DNA band shift assays. DNA band shift assays were carried out essentially as described previously (58). The oligonucleotide used as probe or competitor DNA in band shift assays consisted of the double-stranded sequence 5′ GGATGTTA TAAAGCATGAGTCACTCAGGGGCGCA 3′, corresponding to the AP-1 site in the collagenase promoter (42). Nuclear extracts for band shift analysis were prepared by using a modified Dignam protocol (24). All extracts were standardized for protein concentration before use. Binding reactions were carried out in 30-μl reaction volumes containing 3 μg of nuclear extract protein, 10 fmol of 5′-^{32}P-labeled double-stranded oligonucleotide (approximately 10^6 cpm per reaction), and 1 μg of poly(dI-dC) in 1 mM $MgCl_2$–5 mM dithiothreitol–5 μg of bovine serum albumin (BSA)–0.3 mM phenylmethylsulfonyl fluoride–0.6 mM EDTA–10% glycerol–20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 8.0)–100 mM KCl. Binding reaction mixtures were incubated for 30 min at 23°C. DNA-protein complexes were separated from unbound DNA by electrophoresis in 4% polyacrylamide gels containing 50 mM Tris-HCl (pH 8.0), 200 mM glycine, and 1 mM EDTA and 1× running buffer containing 50 mM Tris-HCl (pH 8.5), 200 mM glycine, and 1 mM EDTA. The gels were dried, audioradiographed, and quantitated by densitometry or phosphor image analysis using ImageQuant software. Where indicated, unlabeled double-stranded competitor DNA was added simultaneously with the labeled oligonucleotide. For antibody competitor assays, specific polyclonal antibodies to JunB, c-Jun, or c-Fos (Santa Cruz Biotechnology), or preimmune sera, were added to the binding reaction mixture prior to addition of labeled oligonucleotide for 15 min at 4°C.

Western blotting (immunoblotting). For Western immunoblot analysis, nuclear lysates were prepared by disruption of cells in 0.5% Nonidet P40–20 mM Tris-HCl (pH 8.0)–15 mM KCl at 4°C. Fifty micrograms of nuclear protein was resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (15% gel) and transferred to nitrocellulose filters (Schleicher & Schuell), using an electroblotting transfer system. Filters were preincubated for 2 h in Tris-buffered saline (TBS) containing 3% BSA at 23°C and then incubated for 3 h with primary antibody to c-Fos or c-Jun protein in TBS–0.5% sodium azide. Filters were washed three times for 10 min each in TBS–0.5% Nonidet P-40 and incubated for 10 min in TBS containing 2% BSA. Filters were then detected by using an enhanced chemiluminescence system (Amersham) and audioradiographed at −70°C.

Construction of recombinant Ad. Construction of the wild-type HBV HBx gene under the control of the cytomegalovirus (CMV) promoter into a replication-defective recombinant Ad vector has been previously described (25). Northern (RNA) blot analysis of HBx mRNAs and biological activity of HBx protein were characterized as described previously (25). All virus stocks were titered on 293 cells before use.

Transcription rates in isolated nuclei. At 12 h after infection, nuclei were

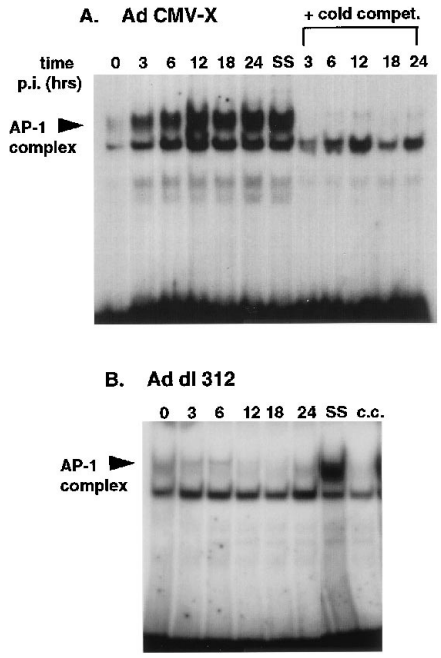

FIG. 2. Induction of AP-1 DNA binding activity by HBx in Chang cells. Serum-starved cells were infected at 25 PFU per cell with Ad CMV-X or control Ad *dl*312. Ad *dl*312 is genetically silent as a result of deletion of region E1 and is phenotypically identical to Ad CMV-Xo. Cells were harvested at the indicated times postinfection (p.i.), extracts were prepared, and equal amounts were used to measure AP-1 DNA binding activity by band shift assay using a ^{32}P-labeled oligonucleotide probe containing one AP-1 binding site. Reactions were carried out with 3 μg of nuclear extract, labeled oligonucleotide, and 1 μg of poly(dI-dC) for 30 min at 23°C. In competition experiments, a 100-fold molar excess of unlabeled oligonucleotide (cold compet.) was added to the binding reactions. Protein-DNA complexes were resolved by electrophoresis on 4% polyacrylamide gels and visualized by autoradiography. Serum stimulation (SS) of cells for 3 h was used as a positive control for induction of AP-1 DNA binding activity.

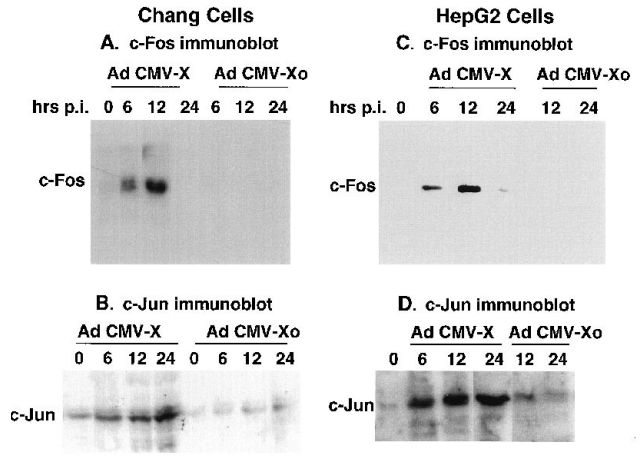

FIG. 3. Enhanced accumulation of endogenous c-Fos and c-Jun proteins by HBx in Chang and HepG2 cells. Serum-starved cells were infected by Ad CMV-X or control Ad CMV-Xo at 25 PFU per cell; then at the indicated times postinfection (p.i.), lysates were prepared as described in Materials and Methods, and equal amounts of protein were resolved by SDS-PAGE (15% gel), transferred to nitrocellulose membranes, and immunoblotted with specific antisera to c-Fos (A and C) or c-Jun (B and D). Immunoblots were detected with an enhanced chemiluminescence system, and autoradiograms were quantitated by densitometry.

isolated from HepG2 cells, using 3×10^7 cells per sample, nuclei were incubated with [α-^{32}P]UTP, and labeled nuclear RNAs were extracted as described previously (68). Slot blot filters containing 500 ng of M13 phage single-stranded cDNAs to Ad early regions E2, E3, and E4 or denatured salmon sperm DNA were prepared, hybridized to 10^6 cpm per sample, and visualized by autoradiography. Probes consist of single-stranded M13 phage DNA containing E2 (Ad type 5 [Ad5] *Bam*HI [59.5 map units {m.u.}]-*Xho*I [70 m.u.]); E3 (Ad5 *Eco*RI [76 m.u.]-*Eco*RI [84 m.u.]); and E4 (Ad5 *Sma*HI [92 m.u.])-*Hin*dIII [97 m.u.]).

Assay of protein kinases. JNK activity was measured by the solid-phase kinase assay using glutathione *S*-transferase (GST)–c-Jun(1-223) as both ligand and substrate essentially as described previously (30). Cell extracts were mixed with GST–c-Jun prebound to glutathione-agarose beads, incubated at 4°C for 3 h, and washed four times, and JNK–c-Jun complexes were retrieved by gentle centrifugation. Beads were incubated in kinase buffer (20 mM Tris-HCl [pH 7.6], 2 mM dithiothreitol, 50 μM ATP, 10 mM β-glycerophosphate, 20 mM *p*-nitrophenylphosphate, 0.5 mM Na_3VO_4, 5 μCi of [γ-^{32}P]ATP) at 30°C for 30 min. Phosphorylated GST–c-Jun was detected by SDS-PAGE (12.5% gel) and quantitated by autoradiography and densitometry. Assay for ERK activity was carried out by phosphorylation of myelin basic protein (MBP) as described previously (7). Briefly, cell extracts were prepared in lysis buffer [20 mM Tris-HCl (pH 8.0), 40 mM NaP_2O_7, 50 mM NaF, 5 mM $MgCl_2$, 100 μM Na_3PO_4, 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 2% Triton X-100, 1% sodium deoxycholate, 6 mM phenylmethylsulfonyl fluoride, 40 μg of leupeptin and aprotinin per ml]. ERK was immunoprecipitated with polyclonal antibodies, resuspended in 20 μl of kinase buffer (40 mM HEPES [pH 7.5], 10 μM ATP, 40 mM $MgCl_2$, 5 μCi of [γ-^{32}P]ATP) and 20 μl of MBP substrate (2 mg/ml; Sigma), incubated for 30 min at 30°C, and resolved by SDS-PAGE (18% gel). Autoradiograms were quantitated by phosphor image analysis or densitometry. Assay for Raf activation was based on the ability of Raf to phosphorylate histone H5 (46). Cells were lysed as for ERK activity, and equal protein amounts of whole cell lysates were subjected to immunoprecipitation with polyclonal Raf antibodies as described above for ERK assays. Immune complexes were washed extensively and incubated as described above, substituting histone H5 protein (Sigma) for MBP and $MnCl_2$ for $MgCl_2$ (46). Reaction mixtures were incubated at 30°C for 20 min and resolved by SDS-PAGE (18% gel), and autoradiograms were quantitated by densitometry. Antibodies to ERKs and Raf were from Upstate Biotechnology. GST–c-Jun was provided by B. Su and M. Karin (University of California, San Diego).

RESULTS

The HBx gene was constructed into a replication-defective Ad vector to permit rapid introduction into cells and for kinetic analysis of AP-1 induction and activation of signalling pathways. Ad-HBx recombinants were shown previously to be replication defective, except in complementing 293 cells that provide E1A and E1B gene products in *trans*, and to produce biologically active HBx protein (7, 8, 25, 38). Several studies previously demonstrated that cotransfected HBx does not activate Ad promoters within the typical time frame of these studies (12 to 24 h), including the major late promoter and early promoters E2 and E4 (6, 65). This issue was examined directly by measuring transcription rates of Ad early regions E2, E3, and E4 in HepG2 cells infected with Ad *dl*309 (wild-type), Ad CMV-X, or control virus Ad CMV-Xo (Fig. 1). Ad CMV-X expresses the HBx gene under the control of the CMV promoter. In Ad CMV-Xo, an HBx mRNA that lacks all AUG codons is expressed (7, 25). Some experiments used Ad *dl*312, a defective virus that lacks region E1, rather than Ad CMV-Xo. The two viruses are phenotypically identical and can be used interchangeably. Transcription runoff assays were performed on nuclei isolated from HepG2 cells at 12 h after infection in the presence of [α-^{32}P]UTP, and the products were hybridized to filters containing probes specific for Ad early regions. Regions E2, E3, and E4 were activated in wild-type Ad-infected cells but not in those infected by Ad CMV-X or the Ad-Xo mutant, which displayed only a very low level of

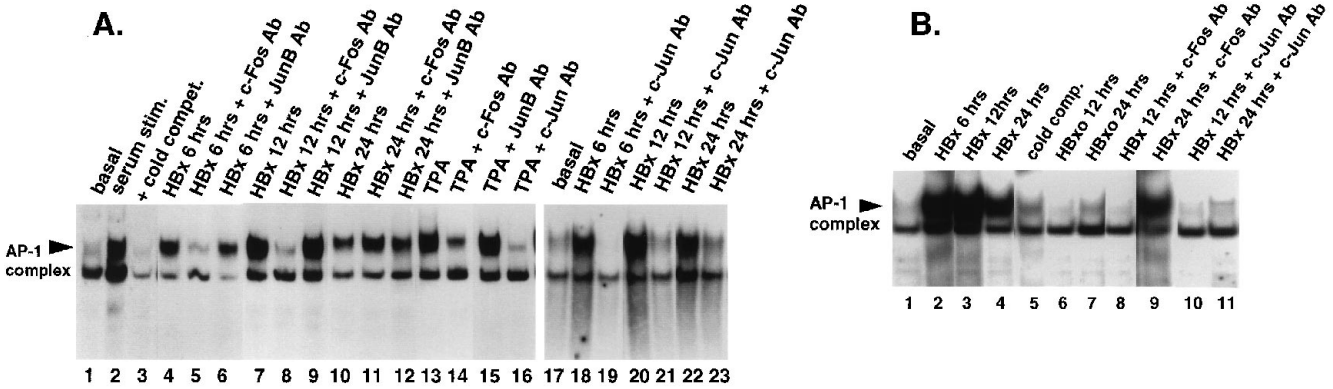

FIG. 4. Changing composition of the AP-1 DNA binding complexes induced during expression of HBx in Chang and HepG2 cells. Serum-starved cells were infected with Ad CMV-X or control Ad CMV-Xo at 25 PFU per cell and harvested at the times indicated, and equal amounts of Chang (A) or HepG2 (B) cell extracts were assayed for AP-1 DNA binding activity by band shift analysis as described in the legend to Fig. 2. Evidence for c-Fos, c-Jun, and JunB participation in AP-1 complex formation was carried out by preincubation of extracts with specific polyclonal blocking antibodies (Ab) corresponding to Fos and Jun proteins, for 15 min at 4°C, prior to addition of labeled oligonucleotide containing an AP-1 binding site. Serum lanes contain preimmune sera as a control. stim., stimulated; cold compet., unlabeled competitor.

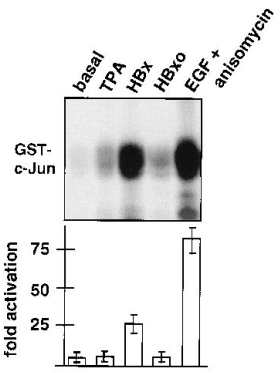

FIG. 5. HBx activates JNK. Serum-starved Chang cells were infected with Ad vectors, or uninfected cells were treated with TPA (20 μM for 30 min) or EGF (50 ng/ml) plus anisomycin (10 μg/ml) for 20 min. At 6 h after infection, cells were lysed and JNK activation was measured by solid-phase assay (30). Whole cell extracts (800 μg) were mixed with (GSH)-agarose beads containing 15 μg of GST–c-Jun, incubated for 3 h, washed well by gentle centrifugation, and incubated with [γ-^{32}P]ATP in kinase buffer for 30 min at 30°C. GST–c-Jun was recovered and analyzed by SDS-PAGE and autoradiography. The mobility of GST–c-Jun protein is indicated. The results of three independent assays were quantitated by phosphor image analysis and presented below a typical experiment with calculated experimental errors.

nonspecific background hybridization. Identical results were obtained from Ad CMV-X-infected Chang cells (data not shown). Therefore, HBx does not induce activation of Ad early promoters during the course of infection typically studied here (12 to 24 h) and does not act by detectably stimulating transcription of Ad genes.

HBx mediates prolonged induction of AP-1 DNA binding complexes. AP-1 DNA binding activity was characterized to determine the composition of the complexes and whether HBx induces a transient or sustained increase in AP-1 complexes. Monolayers of serum-starved cells were infected with either Ad CMV-X or defective control virus Ad *dl*312, which is genetically silent as a result of deletion of region E1A. Nuclear extracts were prepared at various times postinfection. Formation of AP-1 DNA binding complexes was examined by band shift assay using equal amounts of nuclear protein extracts and a ^{32}P-labeled oligonucleotide probe containing one AP-1 binding site (47). HBx induced a strong increase in AP-1 DNA binding activity that was detectable by 3 h after infection, plateaued by 12 h, and remained strongly elevated at 24 h (Fig. 2A). Control viruses Ad *dl*312 and Ad CMV-Xo, which do not express the HBx gene, did not produce an increase in AP-1 DNA binding activity (Fig. 2B). Similar results were also obtained for HBx induction of AP-1 in HepG2 cells (Fig. 4B) and will be described below with respect to the composition of HBx-induced AP-1 DNA binding complexes.

HBx induces AP-1 DNA binding complexes that change in composition during the course of induction. The levels of c-Fos and c-Jun proteins were examined in Chang and HepG2 cells at various times after introduction and expression of HBx by Ad CMV-X. Whole cell extracts were prepared from infected cells, and the levels of Fos and Jun proteins were determined by immunoblot analysis using specific antibodies (Fig. 3). In resting cells, steady-state levels of c-Fos protein are normally undetectable (time zero), as found here (33). After introduction of the HBx gene, a transient increase of c-Fos was observed with similar kinetics in both Chang and HepG2 cells. c-Fos was clearly detectable at 6 h, plateaued to high levels by 12 h, and almost disappeared by 24 h (Fig. 3A and C). The time points chosen for analysis of c-Fos protein levels correspond to weak, strong, and sustained induction of AP-1 DNA binding activity in Chang and HepG2 cells, respectively (Fig. 2A and 4B). There was no detectable induction of c-*fos* in HBxo expressing cells. Northern analysis showed that c-*fos* mRNA levels correlated closely with the accumulation of c-Fos protein (see Fig. 7; also data not shown). In contrast to the transient accumulation of c-Fos protein, induction of c-Jun protein was sustained (Fig. 3B and D). HBx stimulated an approximately two- to threefold increase in c-Jun protein by 6 h, which accumulated another two- to threefold by 24 h (Fig. 3B and D), corresponding to prolonged induction of AP-1 DNA binding complexes shown in Fig. 2A and 4B. In Ad CMV-Xo-infected cells, the level of c-Jun protein remained the same or decreased slightly during the same period, probably because of the reduction in protein synthesis. We therefore investigated whether AP-1 DNA binding complexes induced by HBx change in composition from Fos-Jun heterodimers to c-Jun homodimers, in keeping with the changing abundance of these polypeptides and the sustained synthesis of c-Jun.

Polyclonal antibodies specific to c-Fos, c-Jun, and JunB proteins, or nonspecific antibodies from preimmune sera, were used to block formation of AP-1 DNA binding complexes. Addition of specific antibodies to extracts prior to addition of labeled oligonucleotide probe blocks formation of AP-1 DNA binding complexes by the targeted Fos or Jun polypeptide (48, 53). Antibodies specific to c-Jun protein prevented formation of AP-1 DNA binding complexes induced by HBx at all times tested, from 6 to 24 h after expression of HBx, in both Chang and HepG2 cells (Fig. 4A, lanes 17 to 23; Fig. 4B, lanes 10 and 11). The slight resistance to depletion of AP-1 complexes by c-Jun antibodies at the 12- and 24-h time points in Fig. 4A could be overcome by doubling the amount of antibody used (data not shown). Antibodies to JunB failed to prevent complex formation at any time point tested (Fig. 4A, lanes 6, 9, and 12). The slight depletion of the lower nonspecific band by JunB antibody in Fig. 4A, lane 6, was not a reproducible effect. The quantitative disruption of AP-1 DNA binding complexes by c-Jun and not JunB antibodies indicates that HBx induces AP-1 complexes containing c-Jun protein and not other members of the Jun family. Antibodies to c-Fos protein blocked formation of AP-1 complexes induced in Chang and HepG2 cells by HBx only up to 12 h after infection with Ad CMV-X (Fig. 4A, lanes 5, 8, and 11; Fig. 4B, lanes 8 and 9). AP-1 complexes formed 24 h after HBx expression were not detectably disrupted by antibody to c-Fos (Fig. 4A [compare lanes 5 and 8 with lane 11] and B [compare lane 9 with lane 11]).

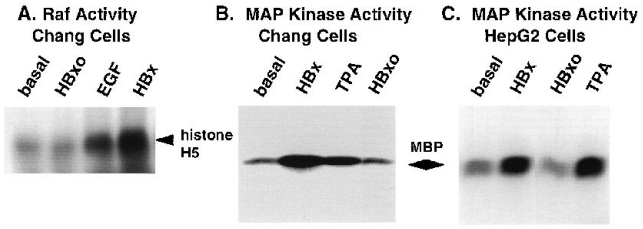

FIG. 6. HBx activation of Raf and ERK. Serum-starved cells were infected with Ad vectors at 25 PFU per cell, or uninfected cells were treated with 50 ng of EGF per ml for 15 min or 20 μM TPA for 30 min. (A) HBx activation of Raf in Chang cells. Cell extracts were prepared at 5 h after infection, and Raf protein was immunoprecipitated and incubated with histone H5 protein and [γ-^{32}P]ATP for 20 min at 30°C. Proteins were resolved by SDS-PAGE (18% gel) and quantitated by densitometry of autoradiograms. (B and C) HBx activation of ERK in Chang cells (B) and HepG2 cells (C). Cells infected with Ad vectors for 5 h or treated with TPA were lysed and immunoprecipitated with polyclonal antibodies to ERK-1 and -2. Immunoprecipitates were incubated with MBP and [γ-^{32}P]ATP at 30°C for 30 min. MBP was resolved by SDS-PAGE, detected, and quantitated by phosphor image analysis.

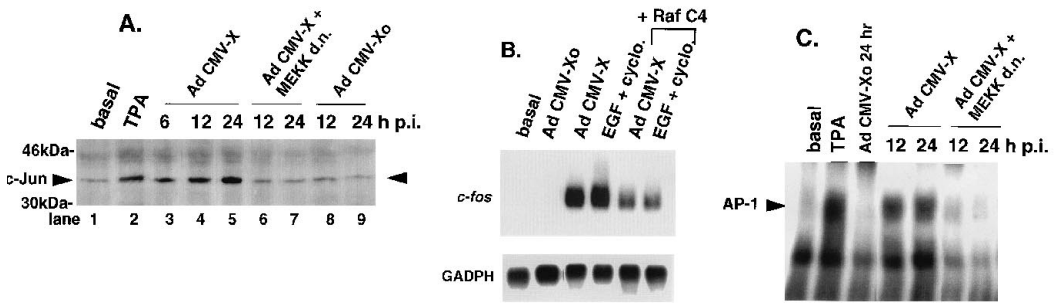

FIG. 7. Effect of MEKK-1 or Raf-C4 catalytic mutant expression on HBx induction of c-*jun* and c-*fos* genes. Serum-starved, subconfluent 10-cm-diameter plates of cells were transfected with 10 μg of plasmids encoding dominant negative (d.n.) mutants of MEKK-1 (43) or Raf-C4 (14) for 5 h and then infected with 25 PFU of Ad CMV-X or Ad CMV-Xo vector per cell as described previously (7, 8). Cells were harvested at the indicated times postinfection (p.i.). Uninfected cells were treated with 20 μM TPA for 30 min or 50 ng of EGF and 10 μg of cycloheximide (cyclo.) per ml for 20 min. (A) Western immunoblot of nuclear c-Jun protein steady-state levels in Chang cells. Equal amounts of nuclear extract were resolved by SDS-PAGE (15% gel), transferred to nitrocellulose, and detected by immunoblot analysis using a polyclonal c-Jun antibody and an enhanced chemiluminescence system. (B) Northern analysis of c-*fos* mRNA levels in Chang cells. Whole cytoplasmic RNAs were prepared, and equal amounts were resolved by formaldehyde-agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to ^{32}P-labeled probes for human c-*fos* or glyceraldehyde-3-phosphate dehydrogenase (GADPH). (C) Formation of AP-1 DNA binding complexes in HepG2 cells coexpressing the MEKK-1 dominant-negative mutant and HBx. Nuclear extracts were prepared at 12 or 24 h postinfection (p.i.), and formation of AP-1 DNA binding complexes was determined by band shift assay as described in the legend to Fig. 2.

These results indicate that AP-1 DNA binding complexes formed during the first 12 h of HBx expression consist of c-Fos–c-Jun heterodimers, whereas complexes formed by 24 hours consist mostly of c-Jun homodimers. Antibodies to Fra-1, a transcription inhibitor of the Fos family, had no effect on complex formation (data not shown), in keeping with the ability of HBx to induce transactivating rather than transrepressing AP-1 complexes. HBx therefore mediates formation of AP-1 DNA binding complexes that change in composition during infection, from c-Fos–c-Jun heterodimers to largely c-Jun–AP-1 dimers. The question of whether c-Jun–AP-1 complexes might also include heterodimers with ATF-2 protein was not addressed in these studies.

HBx induces N-terminal phosphorylation of c-Jun protein. Activation of Ras induces formation of AP-1 DNA binding complexes by stimulating the c-*fos* gene through phosphorylation of the Elk-1/TCF transcription factor (27, 41). In some studies, Ras also promotes N-terminal phosphorylation of c-Jun protein by stimulating JNK activation (9, 40, 55, 63), through a pathway that involves Rac1 and MEKK-1 (18, 44). We previously showed that HBx activates AP-1 in a Ras-dependent manner. Here we show that the Ras-dependent mechanism for HBx stimulation of AP-1 involves activation of two distinct branches of the MAP kinase family comprising ERKs and JNKs.

Activation of de novo c-Jun synthesis has been shown to involve the strong binding and phosphorylation of the N-terminal activation domain by JNKs (2, 9, 37, 51, 59). JNKs are activated independently of ERKs (43–45). To determine whether HBx mediates prolonged c-Jun–AP-1 synthesis by inducing N-terminal phosphorylation of c-Jun, JNK activity was assayed in Chang cell extracts by the solid-phase N-terminal kinase method (30). GST–c-Jun(1-223), containing the N-terminal site of phosphorylation, was immobilized on agarose beads and used as ligand to bind JNK in extracts from cells infected with HBx or HBxo. Control virus Ad-HBxo expresses an HBx mRNA deleted of all AUG codons (7, 25). Uninfected cells treated with anisomycin plus epidermal growth factor (EGF), which very strongly induces JNKs (15) through a Rac1-controlled pathway (18, 43), was used as a positive control. JNK bound to GST–c-Jun beads was retrieved, purified by repeated centrifugation and washing, and incubated with [γ-^{32}P]ATP, and phosphorylation of c-Jun was detected by SDS-PAGE (Fig. 5). At 6 h after introduction, HBx induced a 20- to 25-fold increase in N-terminal c-Jun phosphorylation compared with uninfected, serum-starved cells. Treatment of uninfected cells with EGF plus anisomycin produced an 80-fold increase in JNK activation (one-fourth as much extract was used for this sample). Activation of JNK in the HBxo control was slightly elevated in this experiment, which was not reproducible and was within the experimental error of these assays compared with basal (untreated) controls. The lower band is a degradation product of c-Jun which has also been observed by others. Specificity of JNK activation was demonstrated by the failure to be activated by tetradecanoyl phorbol acetate (TPA) treatment of cells. GST alone is not a substrate for JNK phosphorylation (44, 45) and was not phosphorylated in this assay (data not shown). These data demonstrate that HBx stimulates the activity of the JNKs and subsequent phosphorylation of c-Jun, consistent with the prolonged accumulation of c-Jun protein. A time course analysis showed HBx activation of JNK N-terminal c-Jun phosphorylation at 12 h after HBx expression, but at approximately one-half of the 5-h level (data not shown).

Activation of Raf and ERK-1 and -2 was examined in cells infected with Ad CMV-X or control virus Ad CMV-Xo. Raf activity was measured by the ability of Raf immunoprecipitates to phosphorylate histone H5 protein in vitro (46). Histone H5 protein was resolved by SDS-PAGE, and Raf activity was determined by quantitating the level of histone labeled in autoradiograms (Fig. 6A). Strong Raf activity was evident in immunoprecipitates prepared from cells treated with EGF or expressing HBx but not in HBxo or untreated controls. ERK-1 and -2 activities were independently assessed by immunoprecipitation of extracts with antibodies to ERK-1 and -2, followed by incubation of immunoprecipitates with [γ-^{32}P]ATP and MBP, a substrate of the ERKs. Labeled MBP was resolved by SDS-PAGE. These results show that the ERKs were activated only in cells expressing HBx or treated with TPA but not in controls (Fig. 6B and C). HBx therefore stimulates pathways leading to activation of Raf-ERK and JNK. Collectively, these results demonstrate that HBx activates two different branches of the MAP kinase cascade.

HBx activates c-*jun* through JNK-MEK kinase and activates c-*fos* through Ras-Raf signalling pathways in both Chang and HepG2 cells. Phosphorylation of c-Jun–AP-1 dimers by JNKs

stimulates new synthesis of c-Jun by activating the c-*jun* gene (4). Activation of JNKs involves a pathway controlled by MEKK-1, which is distinct from Ras-Raf activation of ERKs (17, 18, 35, 43). By utilizing a catalytically inactive MEKK-1, we demonstrate here that HBx stimulation of the JNK signalling pathway is essential to maintain prolonged de novo synthesis of c-Jun protein. Quiescent (serum-starved) Chang and HepG2 cells were transfected with a plasmid expressing dominant-negative MEKK-1 and infected 5 h later with Ad CMV-X or Ad CMV-Xo. Infection shortly after transfection drives the uptake of plasmid into the majority of cells in culture (7, 8, 74), thereby leading to expression of the dominant-negative inhibitor in most of the same cells that are infected with the virus vector and express HBx. Previous studies by our laboratory and others have demonstrated the utility of this approach. At various times after infection, cells were harvested and the levels of c-Jun protein present in equal amounts of nuclear Chang cell extracts were determined by SDS-PAGE followed by immunoblot analysis with c-Jun antibodies (Fig. 7A). The identity of c-Jun protein in blots was confirmed by its molecular weight and specific induction by known activators. Controls included uninfected cells with and without exposure to TPA (lanes 1 and 2). HBx induced stimulation of new c-Jun synthesis, assayed between 6 and 24 h (lanes 3 to 5), which was efficiently blocked by prior transfection with the MEKK-1 catalytic mutant (lanes 6 and 7). Cells expressing the HBxo gene did not accumulate additional c-Jun (lanes 8 and 9).

We next determined whether HBx induction of the c-*fos* gene occurred through a Raf-controlled pathway. Chang cells were transfected with a plasmid expressing the Raf-C4 dominant-negative inhibitor and then infected with Ad CMV-X as described above. Induction of the c-*fos* gene was investigated by Northern analysis at 10 h after infection. The Raf-C4 mutant largely (but not completely) blocked HBx activation of c-*fos* (Fig. 7B). The inability of the Raf-C4 mutant to fully suppress accumulation of c-*fos* mRNA probably reflects leakiness of this dominant-negative mutant (7) rather than HBx activation of other c-*fos* stimulatory cascades. Further evidence for prolonged induction of c-Jun by HBx activation of JNK was obtained in HepG2 cells. Cells were transfected with the MEKK-1 dominant-negative mutant and then infected with Ad CMV-X, and formation of AP-1 DNA binding complexes was analyzed by band shift assay (Fig. 7C). Whereas HBx strongly stimulated formation of AP-1 DNA binding complexes measured at 12 and 24 h, complex formation was blocked by coexpression with the MEKK-1 dominant-negative protein. Since c-Fos, c-Jun, and AP-1 DNA binding complexes are present at only very low levels in serum-starved cells, these data collectively demonstrate that HBx induces the c-Jun component of AP-1 by acting on JNK signalling pathways controlled by MEKK-1 and induces the shorter-lived c-Fos component by acting on the Ras-Raf pathway. HBx appears to increase the activities of ERK (7) and JNK (data not shown) rather than abundance.

DISCUSSION

AP-1 DNA binding activity is regulated in a complex manner involving different patterns of Fos and Jun protein expression. The type of AP-1 complex formed partly accounts for the conflicting cell functions associated with induction of the *fos* and *jun* genes, which range from proliferation and transformation to differentiation and growth arrest (reviewed in reference 33). We therefore characterized (i) the signalling pathways and the mechanism by which HBx activates AP-1 and (ii) the types of complexes induced. HBx was found to mediate a prolonged accumulation of AP-1 DNA binding complexes in Chang cells (Fig. 2A) and in HepG2 cells (Fig. 4B), which were sustained for at least 24 h, beyond which activation could not be reliably analyzed. Characterization of AP-1 complexes induced by HBx was performed with blocking antibodies directed to different members of the Fos and Jun family (Fig. 4). HBx first induced the transient accumulation of c-Fos–c-Jun heterodimers in Chang and HepG2 cells between 3 and 12 h, which was replaced by the marked accumulation of c-Jun homodimers between 12 to 24 h. HBx induced de novo synthesis of c-Fos protein consistent with its transient participation in AP-1 complex formation (Fig. 3). Studies have shown that the ERKs mediate activation of the c-*fos* gene in a Raf-dependent pathway (30, 70). We previously showed that HBx induces activation of the Ras-Raf-MAP kinase cascade (7, 25), consistent with the ability to mediate de novo synthesis of c-Fos. In this report, we further demonstrate that HBx rapidly activates the ERK members of the MAP kinases, with kinetics consistent with stimulation of the c-*fos* gene (Fig. 6), in differentiated (HepG2) and poorly differentiated (Chang) hepatocyte cell lines. Moreover, a catalytic mutant of Raf blocked HBx induction of the c-*fos* gene (Fig. 7). A surprising result of this study was the prolonged induction by HBx of high levels of c-Jun–AP-1 complexes, which largely replaced c-Fos–c-Jun complexes by 24 h after HBx expression (Fig. 4).

Continued accumulation of active c-Jun protein involves stimulation of c-*jun* transcription and posttranslational control by phosphorylation. Transcription of the c-*jun* gene is strongly stimulated by EGF plus anisomycin, or UV light, and in some cell systems it is moderately stimulated by activation of Ras (22). New c-Jun synthesis involves binding of active c-Jun homodimers (3, 20) or c-Jun–ATF-2 heterodimers to the c-Jun promoter (67). HBx has been shown to interact with transcription factor ATF/CREB (39) and alter its binding specificity (72). Whether de novo synthesis of c-Jun protein induced by HBx involves formation of c-Jun–ATF-2 complexes, and whether these complexes might persist in HBx-stimulated cells, has not yet been determined. It is clear, though, that HBx induces the c-*jun* gene by activation of JNK signal transduction cascades.

The transcriptional activation of the c-*jun* gene is very strongly elevated by N-terminal phosphorylation of c-Jun protein carried out by the JNKs (21, 22, 31, 35, 45, 57, 71). Using a solid-phase assay for c-Jun N-terminal binding and phosphorylation, we provided direct evidence for HBx stimulation of JNK activity (Fig. 5), by acting on JNK signalling pathways controlled by MEKK-1 (Fig. 7). Temporally, HBx activated the JNKs rapidly, with kinetics similar to that for ERK activation (data not shown). HBx induced moderate (20- to 25-fold) activation of the JNKs, similar to that observed for activation by Ras without Rac1 involvement (18, 45) but considerably lower than the strong activation (~80-fold) observed for EGF plus anisomycin (Fig. 5) (15, 43–45), which acts through a Rac1-controlled pathway (18, 43). It therefore seems likely that HBx activation of JNK occurs through a Ras–MEKK-1 pathway that does not involve Rac1, although this point needs to be explored further. The level and kinetics of JNK activation by HBx are also consistent with the strong induction of AP-1 DNA binding activity observed. HBx activation of c-*fos* was found to occur through a Ras-Raf pathway, as expected (Fig. 7). Collectively, these data demonstrate that HBx activates AP-1 by rapidly inducing activation of ERK and JNK MAP kinases. We previously showed that HBx activation of Ras is essential for its activation of MAP kinases and AP-1 DNA binding activity (7). These results suggest that HBx stimulates

Ras-dependent signals that transduce independent activation of two distinct branches of the MAP kinase cascade.

There are other reports of AP-1 DNA binding complexes changing during the course of induction from dimeric c-Fos–c-Jun to c-Jun complexes, as observed here. These include AP-1 complexes induced by thrombin (62), tumor necrosis factor alpha (13), serum stimulation of rat and chicken fibroblasts (29), and predifferentiation growth arrest in keratinocytes by various agents (10). In all cases, it is believed that prolonged accumulation of c-Jun–AP-1 complexes results from autostimulation of the c-*jun* gene. The c-Jun–AP-1 dimers observed here accumulated to levels similar to those observed in previous studies, which were sufficient to influence cellular transcriptional activity.

Activation of Ras transduces signals to multiple targets in mammalian cells. Moreover, Ras possesses at least two separate activities for transformation, one of which includes activation of Raf and ERKs (70). The nature of the other signal(s) is not known but likely includes N-terminal phosphorylation of c-Jun and activated JNKs, at least in some cells (70). Thus, since HBx can stimulate cell proliferation (7) and rapid progression through the cell cycle in a Ras-dependent manner (8), it now needs to be determined whether HBx induction of cell proliferation involves activation of the ERK and JNK branches of the MAP kinase pathway and whether these activities occur in the context of an HBV infection.

ACKNOWLEDGMENTS

We thank L. Khatri for technical assistance during parts of this work.

M.D. was supported by a fellowship from the Associazione Italiana per la Ricerca sul Cancro. This work was supported by Public Health Service grants CA54525 and CA56533 to R.J.S. from the National Cancer Institute.

REFERENCES

1. **Alani, R., P. Brown, B. Binetruy, H. Dosaka, R. K. Rosenberg, P. Angel, M. Karin, and M. J. Birrer.** 1991. The transactivating domain of the c-Jun proto-oncogene is required for cotransformation of rat embryo cells. Mol. Cell. Biol. **11:**6286–6295.
2. **Alvarez, E., I. C. Northwood, F. A. Gonzalez, D. A. Latour, A. Seth, C. Abate, T. Curran, and R. J. Davis.** 1991. Pro-Leu-Ser/Thr-Pro is a consensus primary sequence for substrate protein phosphorylation. J. Biol. Chem. **266:** 15277–15285.
3. **Angel, P., E. A. Allegretto, S. Okino, K. Hattori, W. J. Boyle, T. Hunter, and M. Karin.** 1988. Oncogene jun encodes a sequence specific transactivator similar to AP1. Nature (London) **332:**166–171.
4. **Angel, P., K. Hattori, T. Smeal, and M. Karin.** 1988. The *jun* proto-oncogene is positively autoregulated by its product, Jun-AP-1. Cell **55:**875–885.
5. **Angel, P., and M. Karin.** 1991. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim. Biophys. Acta **1072:**129–157.
6. **Aufiero, B., and R. J. Schneider.** 1990. The hepatitis B virus X-gene product trans-activates both RNA polymerase II and III promoter. EMBO J. **9:**497–504.
7. **Benn, J., and R. J. Schneider.** 1994. Hepatitis B virus HBx protein activates Ras-GTP complex formation and establishes a Ras, Raf, MAP kinase signalling cascade. Proc. Natl. Acad. Sci. USA **91:**10350–10354.
8. **Benn, J., and R. J. Schneider.** 1995. Hepatitis B virus HBx protein deregulates cell cycle checkpoint controls. Proc. Natl. Acad. Sci. USA **92:**11215–11219.
9. **Binetruy, B., T. Smeal, and M. Karin.** 1991. H-Ras augments c-Jun activity and stimulates phosphorylation of its activation domain. Nature (London) **351:**122–127.
10. **Blatti, S. P., and R. E. Scotte.** 1992. Stable induction of c-jun mRNA expression in normal human keratinocytes by agents that induce predifferentiation growth arrest. Cell Growth Differ. **3:**429–434.
11. **Bohman, D., T. J. Bos, A. Admon, T. Nishimura, P. K. Vogt, and R. Tijan.** 1987. Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. Science **238:**1386–1392.
12. **Boyle, W. J., T. Smeal, L. H. K. Defize, P. Angel, J. R. Woodgett, M. Karin, and T. Hunter.** 1991. Activation of protein kinase C decreases phosphorylation of c-jun at sites that negatively regulate its DNA binding activity. Cell **64:**573–584.
13. **Brenner, D. A., M. O'Hara, P. Angel, M. Chojkier, and M. Karin.** 1989. Prolonged activation of jun and collagenase genes by tumor necrosis factor alpha. Nature (London) **337:**661–663.
14. **Bruder, J. T., G. Heidecker, and U. R. Rapp.** 1992. Serum-, TPA-, and Ras-induced expression from Ap-1/Ets-driven promoters requires Raf-1 kinase. Genes Dev. **6:**545–556.
15. **Cano, E., C. A. Hazzalin, and L. C. Mahadevan.** 1994. Anisomycin-activated protein kinases p45 and p55 but not mitogen-activated protein kinases ERK-1 and -2 are implicated in the induction of c-*fos* and c-*jun*. Mol. Cell. Biol. **14:**7352–7362.
16. **Cheong, J.-H., M.-K. Yi, Y. Lin, and S. Murakami.** 1995. Human RPB5, a subunit shared by eukaryotic nuclear polymerases, binds human hepatitis B virus X protein and may play a role in X transactivation. EMBO J. **14:**143–150.
17. **Cosco, O. A., M. Chiarello, G. Kalinec, J. M. Kyriakis, J. Woodgett, and J. S. Gutkind.** 1995. Transforming G-protein coupled receptors potently activate JNK (SAPK): evidence for a divergence from the tyrosine kinase signaling pathway. J. Biol. Chem. **270:**5620–5624.
18. **Cosco, O. A., M. Chiariello, J.-C. Yu, H. Teramoto, P. Crespo, N. Xu, T. Miki, and J. S. Gutkind.** 1995. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell **81:** 1137–1146.
19. **Cross, J. C., P. Wen, and W. J. Rutter.** 1993. Transactivation by hepatitis B virus X protein is promiscuous and dependent on mitogen activated cellular serine/threonine kinases. Proc. Natl. Acad. Sci. USA **90:**8078–8082.
20. **Deng, T., and M. Karin.** 1994. c-Fos transcriptional activity stimulated by H-Ras-activated protein kinase distinct from JNK and ERK. Nature (London) **371:**171–175.
21. **Derijard, B., M. Hibi, I.-H. Wu, T. Barrett, B. Su, T. Deng, M. Karin, and R. J. Davis.** 1994. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell **76:**1025–1037.
22. **Devary, Y., R. A. Gottlieb, T. Smeal, and M. Karin.** 1992. The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases. Cell **71:**1081–1091.
23. **deVries-Smits, A. M., B. M. T. Burgering, S. J. Leevers, C. J. Marshall, and J. L. Bos.** 1992. Involvement of p21ras in activation of extracellular regulated kinase 2. Nature (London) **357:**602–604.
24. **Dignam, J., R. Lebovitz, and R. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
25. **Doria, M., N. Klein, R. Lucito, and R. J. Schneider.** 1995. Hepatitis B virus HBx protein is a dual specificity cytoplasmic activator of Ras and nuclear activator of transcription factors. EMBO J. **14:**4747–4757.
26. **Faktor, O., and Y. Shaul.** 1990. The identification of hepatitis B virus X gene responsive elements reveals functional similarity of X and HTLV-I tax. Oncogene **5:**867–872.
27. **Gille, H., A. Sharrocks, and P. Shaw.** 1992. Phosphorylation of p62TCF by MAP kinase stimulates ternary complex formation at cFos promoter. Nature (London) **358:**414–417.
28. **Haviv, I., D. Vaizel, and Y. Shaul.** 1995. The X protein of hepatitis B virus coactivates potent activation domains. Mol. Cell. Biol. **15:**1079–1085.
29. **Hawker, K. L., A. Pintzas, R. F. Hennigan, D. F. Gillespie, and B. W. Ozanne.** 1993. Transformation by the *fos* or *jun* oncogene does not increase AP-1 DNA-binding activity. J. Virol. **67:**5487–5495.
30. **Hibi, M., A. Lin, T. Smeal, A. Minden, and M. Karin.** 1993. Identification of an oncoprotein- and UV-responsive protein kinase that bind and potentiate the c-Jun activation domain. Genes Dev. **7:**2135–2148.
31. **Hu, K.-Q., J. M. Vierling, and A. Siddiqui.** 1990. Trans-activation of HLA-DR gene by hepatitis B virus X gene product. Proc. Natl. Acad. Sci. USA **87:**7140–7144.
32. **Kallunki, T., B. Su, I. Tsigelny, H. K. Sluss, B. Derijard, G. Moore, R. Davis, and M. Karin.** 1994. JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation. Genes Dev. **8:**2996–3007.
33. **Karin, M.** 1991. The AP-1 complex and its role in transcriptional control by protein kinase C, p. 143–161. *In* P. Cohen and G. Foulkes (ed.), Molecular aspects of cellular regulation, vol. 6. Elsevier North-Holland Biomedical Press, Amsterdam.
34. **Kekule, A. S., U. Lauer, L. Weiss, B. Luber, and P. H. Hofschneider.** 1993. Hepatitis B virus transactivator HBx uses a tumor promoter signalling pathway. Nature (London) **361:**742–745.
35. **Kyriakis, J. M., P. Banerjee, E. Nikolakaki, T. Dai, E. A. Rubie, M. F. Ahmad, J. Avruch, and J. R. Woodgett.** 1994. The stress-activated protein kinase subfamily of c-Jun kinases. Nature (London) **369:**156–160.
36. **Levrero, M., C. Balsano, G. Natoli, M. L. Avantaggiati, and E. Elfassi.** 1990. Hepatitis B virus X protein transactivates the long terminal repeats of human immunodeficiency virus types 1 and 2. J. Virol. **64:**3082–3086.
37. **Lin, A., J. Frost, T. Deng, N. Al-Alawi, T. Smeal, U. Kikkawa, T. Hunter, D. Brenner, and M. Karin.** 1992. Casein kinase II is a negative regulator of c-Jun DNA binding and AP-1 activity. Cell **70:**777–789.
38. **Lucito, R., and R. J. Schneider.** 1992. Hepatitis B virus X protein activates

transcription factor NF-κB without a requirement for protein kinase C. J. Virol. **66:**983–991.
39. **Maguire, H. F., J. P. Hoeffler, and A. Siddiqui.** 1991. HBV X protein alters the DNA binding specificity of CREB and ATF-2 by protein-protein interactions. Science **252:**842–844.
40. **Mahe, Y., N. Mukaida, K. Kuno, M. Akiyama, N. Ikeda, K. Matshushima, and S. Murakami.** 1991. Hepatitis B virus X protein transactivates human interleukin-8 gene through acting on nuclear factor κB and CCAAT/enhancer-binding protein-like cis elements. J. Biol. Chem. **266:**13759–13763.
41. **Marais, R., J. Wynne, and R. Treisman.** 1993. The SRF accessory protein Elk-1 contains a growth factor transcriptional activation domain. Cell **73:** 381–393.
42. **Meyer, M., W. H. Caselmann, V. Schluter, R. Schreck, P. H. Hofschneider, and P. A. Baeuerle.** 1992. Hepatitis B virus transactivator MHBst: activation of NF-κB, selective inhibition by antioxidants and integral membrane localization. EMBO J. **11:**2991–3001.
43. **Minden, A., A. Lin, F. X. Claret, A. Abo, and M. Karin.** 1995. Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and CDC42Hs. Cell **81:**1147–1157.
44. **Minden, A., A. Lin, M. McMahon, C. Lange-Carter, B. Derijard, R. J. Davis, G. L. Johnson, and M. Karin.** 1994. Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. Science **266:**1719–1723.
45. **Minden, A., A. Lin, T. Smeal, B. Derijard, M. Cobb, R. Davis, and M. Karin.** 1994. c-Jun N-terminal phosphorylation correlates with activation of the JNK subgroup but not the ERK subgroup of mitogen-activated protein kinases. Mol. Cell. Biol. **14:**6683–6688.
46. **Moelling, K., B. Heimann, P. Beimling, U. R. Rapp, and T. Sander.** 1984. Serine- and threonine-specific protein kinase activities of purified gag-mil and gag-raf proteins. Nature (London) **312:**558–561.
47. **Muller, U., M. P. Roberts, D. A. Engel, W. Doerfler, and T. Shenk.** 1989. Induction of transcription factor AP-1 by adenovirus E1A protein and cAMP. Genes Dev. **3:**1991–2002.
48. **Nakabeppu, Y., K. Ryder, and D. Nathans.** 1988. DNA binding activities of three murine Jun proteins: stimulation by Fos. Cell **55:**907–915.
49. **Natoli, G., M. L. Avantaggiati, P. Chirillo, A. Costanzo, M. Artini, C. Balsano, and M. Levrero.** 1994. Induction of the DNA-binding activity of c-Jun/c-Fos heterodimers by the hepatitis B virus transactivator pX. Mol. Cell. Biol. **14:**989–998.
50. **Natoli, G., M. L. Avantaggiati, P. Chirillo, P. L. Puri, A. Ianni, C. Balsano, and M. Levrero.** 1994. Ras- and raf-dependent activation of c-jun transcriptional activity by the hepatitis B virus transactivator pX. Oncogene **9:**2837–2843.
51. **Pulverer, B. J., J. M. Kryakis, J. Avruch, E. Nikolakakie, and J. Woodgett.** 1991. Phosphorylation of c-jun mediated by MAP kinases. Nature (London) **353:**670–674.
52. **Qadri, I., H. F. Maguire, and A. Siddiqui.** 1995. Hepatitis B virus transactivator protein X interacts with the TATA-binding protein. Proc. Natl. Acad. Sci. USA **92:**1003–1007.
53. **Rauscher, F. R., L. C. Sambucetti, T. Curran, R. J. Distel, and B. M. Spiegelman.** 1988. Common DNA binding site for Fos protein complexes and transcription factor AP-1. Cell **52:**471–480.
54. **Seto, E., P. J. Mitchell, and T. S. B. Yen.** 1990. Transactivation by the hepatitis B virus X protein depends on AP-2 and other transcription factors. Nature (London) **344:**72–74.
55. **Seto, E., T. S. B. Yen, B. M. Peterlin, and J.-H. Ou.** 1988. Trans-activation of the human immunodeficiency virus long terminal repeat by the hepatitis B virus X protein. Proc. Natl. Acad. Sci. USA **85:**8290–8286.
56. **Siddiqui, A., R. Gaynor, A. Srinivasan, J. Mapoles, and R. W. Farr.** 1989. Trans-activation of viral enhancers including long terminal repeat of the human immunodeficiency virus by the hepatitis B virus X protein. Virology **169:**479–484.
57. **Sluss, H. K., T. Barrett, B. Derijard, and R. J. Davis.** 1994. Signal transduction by tumor necrosis factor mediated by JNK protein kinases. Mol. Cell. Biol. **14:**8376–8384.
58. **Smeal, T., P. Angel, J. Meek, and M. Karin.** 1989. Different requirements for formation of Jun:Jun and Jun:Fos complexes. Genes Dev. **3:**2091–2100.
59. **Smeal, T., D. Binetruy, M. Birrer, and M. Karin.** 1991. Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of cJun on serines 63 and 73. Nature (London) **354:**494–496.
60. **Su, F., R. Lucito, and R. J. Schneider.** Unpublished data.
61. **Thomas, S. M., M. DeMarco, G. D'Arcangelo, S. Halegoua, and J. S. Brugge.** 1992. Ras is essential for nerve growth factor and phorbol ester induced tyrosine phosphorylation of MAP kinases. Cell **68:**1031–1040.
62. **Trejo, J., J.-C. Chambard, M. Karin, and J. H. Brown.** 1992. Biphasic increase in c-*jun* mRNA is required for induction of AP1-mediated gene transcription: differential effects of muscarinic and thrombin receptor activation. Mol. Cell. Biol. **12:**4742–4750.
63. **Twu, J.-S., M.-Y. Lai, D.-S. Chen, and W. S. Robinson.** 1993. Activation of protooncogene c-jun by the X protein of hepatitis B virus. Virology **192:**346–350.
64. **Twu, J.-S., and W. S. Robinson.** 1989. Hepatitis B virus X gene can transactivate heterologous viral sequences. Proc. Natl. Acad. Sci. USA **86:**2046–2050.
65. **Twu, J. S., and R. H. Schloemer.** 1987. Transcriptional *trans*-activating function of hepatitis B virus. J. Virol. **61:**3448–3453.
66. **Unger, T., and Y. Shaul.** 1990. The X protein of the hepatitis B virus acts as a transcription factor when targeted to its responsive element. EMBO J. **9:**1889–1895.
67. **van Dam, H., M. Duyndam, R. Rottier, A. Bosch, L. de Vries-Smits, P. Herrlich, A. Zantema, P. Angel, and A. J. van der Eb.** 1993. Heterodimer formation of c-Jun and ATF-2 is responsible for induction of c-Jun by the 243 amino acid adenovirus E1A protein. EMBO J. **12:**479–487.
68. **Velcich, A., and E. B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature (London) **311:**433–438.
69. **Wang, H.-D., C.-H. Yuh, C. V. Dang, and D. L. Johnson.** 1995. The hepatitis B virus X protein increases the cellular level of TATA-binding protein which mediates transactivation of RNA polymerase III genes. Mol. Cell. Biol. **15:**6720–6728.
70. **Westwick, J. K., A. D. Cox, C. J. Der, M. H. Cobb, M. Hibi, M. Karin, and D. A. Brenner.** 1994. Oncogenic Ras activates c-Jun via a separate pathway from the activation of extracellular signal-regulated kinases. Proc. Natl. Acad. Sci. USA **91:**6030–6034.
71. **Westwick, J. K., C. Weitzel, A. Minden, M. Karin, and D. A. Brenner.** 1994. Tumor necrosis factor alpha stimulates AP-1 activity through prolonged activation of the c-Jun kinase. J. Biol. Chem. **269:**26396–26401.
72. **Williams, J. S., and O. M. Andrisani.** 1995. The hepatitis B virus X protein targets the basic region-leucine zipper domain of CREB. Proc. Natl. Acad. Sci. USA **92:**3819–3823.
73. **Wood, K. W., C. Sarnecki, T. M. Roberts, and J. Blenis.** 1992. ras mediates nerve growth factor receptor modulation of three signal transducing protein kinases: MAP kinase, Raf-1 and RSK. Cell **68:**1041–1050.
74. **Yoshimura, K., M. A. Rosenfeld, P. Seth, and R. G. Crystal.** 1993. Adenovirus-mediated augmentation of cell transfection with unmodified plasmid vectors. J. Biol. Chem. **268:**2300–2303.
75. **Zahm, P., P. H. Hofschneider, and R. Koshy.** 1988. The HBV X-ORF encodes a transactivator: a potential factor in viral hepatocarcinogenesis. Oncogene **3:**169–177.
76. **Zhou, D.-X., A. Taraboulous, J.-H. Ou, and T. S. B. Yen.** 1990. Activation of class I major histocompatibility complex gene expression by hepatitis B virus. J. Virol. **64:**4025–4028.