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Hepatitis B virus HBx protein activates Ras–GTP complex formation and establishes a Ras, Raf, MAP kinase signaling cascade

(mitogen-activated protein kinase/cell proliferation)

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ABSTRACT **Hepatitis B virus produces a small (154-amino acid) transcriptional transactivating protein, HBx, which is required for viral infection and has been implicated in virus-mediated liver oncogenesis. However, the molecular mechanism for HBx activity and its possible influence on cell proliferation have remained obscure. A number of studies suggest that HBx may stimulate transcription by indirectly activating transcription factors, possibly by influencing cell signaling pathways. We now present biochemical evidence that HBx activates Ras and rapidly induces a cytoplasmic signaling cascade linking Ras, Raf, and mitogen-activated protein kinase (MAP kinase), leading to transcriptional transactivation. HBx strongly elevates levels of GTP-bound Ras, activated and phosphorylated Raf, and tyrosine-phosphorylated and activated MAP kinase. Transactivation of transcription factor AP-1 by HBx is blocked by inhibition of Ras or Raf activities but not by inhibition of Ca^{2+}- and diacylglycerol-dependent protein kinase C. HBx was also found to stimulate DNA synthesis in serum-starved cells. The hepatitis B virus HBx protein therefore stimulates Ras–GTP complex formation and promotes downstream signaling through Raf and MAP kinases, and may influence cell proliferation.**

The hepatitis B virus HBx protein is a transactivator of transcription (1–3) which activates the specific RNA polymerase II transcription factors AP-1 (refs. 4–7; J.B., R. Lucito, M. Doria, and R.J.S., unpublished work), AP-2 (4), and NF-κB (9–12) and RNA polymerase III transcription factor TFIIIC (13). HBx is also required to establish woodchuck hepatitis virus infection in woodchucks (14, 15). Although HBx has been implicated in hepatitis B virus-induced hepatocellular carcinoma (16), this point remains controversial (17, 18).

The mechanism by which HBx induces transcriptional transactivation is unknown. A few studies have suggested that HBx might perhaps bind to and directly activate certain transcription factors (19–21), although this has not been proven. HBx does not possess significant structural or sequence homologies with other known transactivators, nor does it bind DNA directly (unpublished observations). HBx contains a short sequence of limited homology to Kunitz-like protease inhibitors (22), but such an activity has not been convincingly demonstrated for HBx. In contrast, several studies have suggested that HBx most likely acts indirectly, since a variety of transcription factors are activated by the protein (6, 13, 23). Thus, HBx could conceivably act on components of cellular signal transduction pathways. In this regard, most studies found no requirement for Ca^{2+}/diacylglcerol-dependent protein kinase C (PKC) activity in mediating HBx transactivation (7, 12, 24, 25), although there is one report of such a requirement (6).

We have explored the ability of HBx to influence cell signal transduction pathways. We particularly focused on the Ras, Raf, mitogen-activated protein kinase (MAP kinase) pathway because its activation could explain many of the transactivational activities ascribed to HBx and because some evidence has been presented that HBx-induced transactivation may involve Raf activity (24). We now show that HBx induces a high level of Ras activity by stimulating Ras–GTP cycling, which transduces its signal downstream, activating Raf and MAP kinase activities. Establishment of this cascade is also shown to be vital for HBx transactivational activity and to lead to an increase in cellular DNA synthesis.

MATERIALS AND METHODS

Ras Activation Assays. Chang cells were serum starved by culture in Dulbecco's modified Eagle's medium (DMEM) with 0.5% calf serum for 48 hr, infected by incubation for 3 hr with recombinant HBx-expressing adenovirus (Ad-X) vectors at 25 plaque-forming units per cell, and labeled with [^{32}P]orthophosphate (0.5 mCi/ml; 1 Ci = 37 GBq) for 3 hr in phosphate-free DMEM with 2% calf serum. Uninfected cells were stimulated with phorbol 12-myristate 13-acetate ["12-*O*-tetradecanoylphorbol 13-acetate" (TPA), 50 ng/ml] for 5 min during [^{32}P]orthophosphate labeling. Cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and lysed by 30 min of incubation at 4°C in lysis buffer [50 mM Hepes, pH 7.4/1% (vol/vol) Triton X-100/100 mM NaCl/5 mM $MgCl_2$/1 mM Na_3VO_4/10 mM NaF/1 mM phenylmethanesulfonyl fluoride (PMSF) with aprotinin (20 μg/ml) and leupeptin (10 μg/ml)]. Lysates were clarified by microcentrifugation for 3 min at 10,000 × *g* at 4°C, the supernatant was precleared with protein G-Sepharose, and immunoprecipitation was performed for 60 min at 4°C with Ras antibody Y13-259 (26) (a gift from A. Pelicer, New York University) at 5 μg per sample. Y13-259 was omitted from control samples. Immune complexes were collected by precipitation with protein G-Sepharose and washed eight times in 50 mM Tris·HCl, pH 7.5/20 mM $MgCl_2$. Bound nucleotides were eluted by incubation for 20 min at 65°C in 20 mM Tris·HCl, pH 7.5/20 mM EDTA/2% SDS. Nucleotides were separated by thin-layer chromatography (TLC) on PEI-cellulose plates in 0.75 M KH_2PO_4 (pH 3.5), visualized by autoradiography and quantitated by scintillation counting of spots from the TLC plate. Phosphate contents were corrected by factors of 1/3 for GTP and 1/2 for GDP. Percent GTP–Ras was calculated from the ratio of corrected values for GTP/(GTP + GDP). Data shown are the average of three independent experiments.

Transfection and Infection of Cells. Chang cells at 30% confluence were transfected with DNA plasmids by calcium

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Abbreviations: Ad, adenovirus; MAP kinase, mitogen-activated protein kinase; PKC, protein kinase C; PMSF, phenylmethanesulfonyl fluoride; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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phosphate precipitation using 20 μg of plasmid DNA per 10-cm plate. Plasmids consisted of the Ras dominant negative mutant Asn-17 (27), Raf dominant negative mutant C4 (28), and pCMV-HBx or pCMV-HBx$_0$ (J.B. *et al.*, unpublished work). Where indicated, cells were infected with Ad-X recombinant viruses or treated with TPA and/or calphostin C as described above. Transfection efficiencies were typically in the range of 80% of cells. This was determined by cotransfection of a plasmid encoding β-galactosidase and staining for activity in cells *in situ*. We have found that these high efficiencies are typical of Chang liver cells when transfected at low cell density.

Band-Shift Assays. Electrophoretic band-shift assays were carried out essentially as described (29). Double-stranded oligodeoxynucleotides for probe or competitor corresponded to the AP-1 site in the collagenase promoter: 5′-GGATGTTATAAAGCATGAGTCACTCAGGGGCGCA-3′. Nuclear extract was prepared by the modified Dignam *et al.* protocol (30) and 3 μg of protein was used for binding reactions in 30 μl containing 10 fmol of ^{32}P-5′-end-labeled double-stranded oligodeoxynucleotide (≈10^6 cpm per reaction), 1 μg of poly[d(I-C)], 1 mM $MgCl_2$, 5 mM dithiothreitol, 5 μg of bovine serum albumin, 0.3 mM PMSF, 0.6 mM EDTA, 10% (vol/vol) glycerol, 20 mM Hepes (pH 8.0), and 100 mM KCl. Reaction mixtures were incubated for 30 min at 23°C. DNA–protein complexes were resolved by electrophoresis in 4% polyacrylamide gels containing 50 mM Tris·HCl (pH 8.0), 200 mM glycine, and 1 mM EDTA; electrophoresis buffer contained 50 mM Tris·HCl (pH 8.5), 200 mM glycine, and 1 mM EDTA. Gels were dried and autoradiographed, and bands were quantitated by densitometry. Where indicated, unlabeled double-stranded competitor was added simultaneously with the labeled oligonucleotide.

Immunoprecipitation and Immunoblot Analyses. Lysates were prepared in 20 mM Tris·HCl, pH 8.0/40 mM $Na_4P_2O_7$/50 mM NaF/5 mM $MgCl_2$/100 μM Na_3VO_4/10 mM EGTA/2% Triton X-100/1% sodium deoxycholate/0.2% SDS/6 mM PMSF with leupeptin (40 μg/ml) and aprotinin (40 μg/ml). For each sample, 200 μg of protein lysate was subjected to SDS/10% PAGE, transferred to nitrocellulose, and immunoblotted with antibody to p42 MAP kinase or p42/p44 MAP kinase. Immune complexes were visualized with the ECL chemiluminescence system (Amersham). For assay of MAP kinase activity, MAP kinase was immunoprecipitated from cell lysates prepared in the buffer described above. Immunoprecipitates were suspended in 20 μl of myelin basic protein substrate (2 mg/ml; Sigma) and 20 μl of reaction mixture containing 5 μCi of [γ-^{32}P]ATP, 10 μM ATP, 40 mM $MgCl_2$, and 40 mM Hepes (pH 7.5) (31). After incubation for 30 min at 30°C, reactions were stopped by

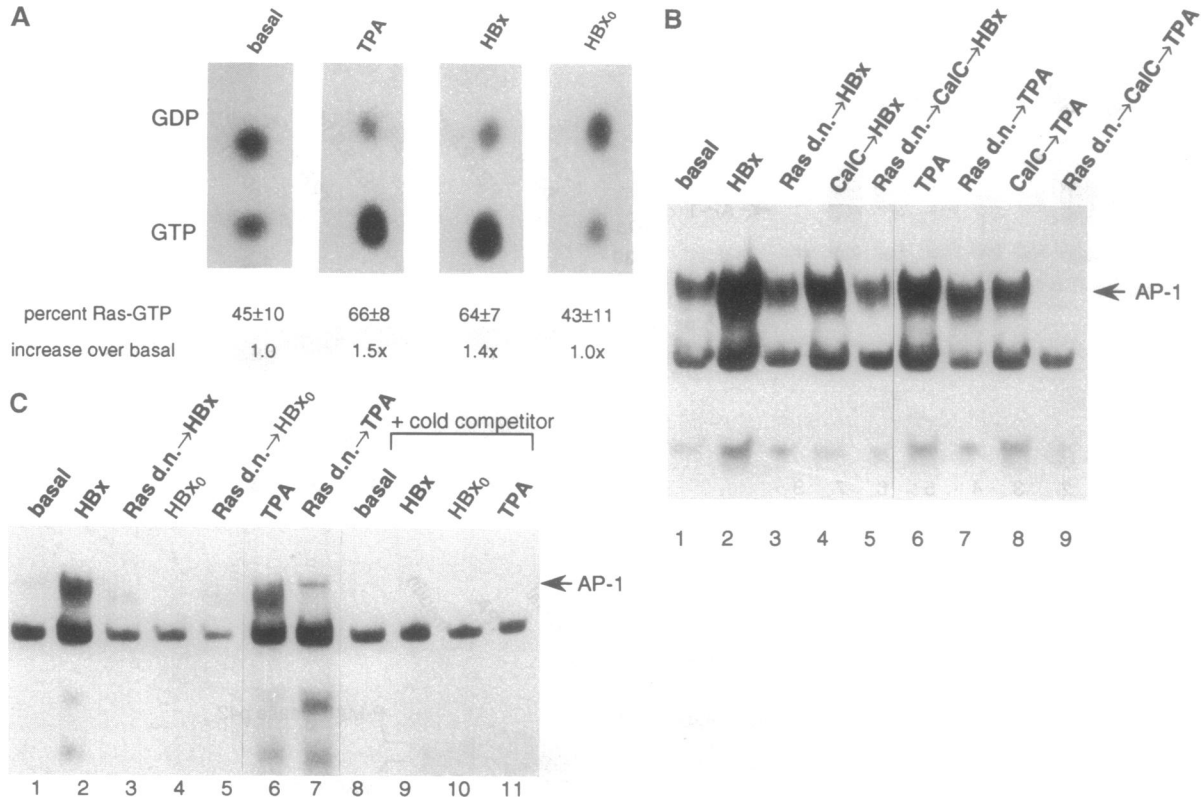

FIG. 1. Effect of HBx on Ras activation. (*A*) HBx-induced increase in GTP-bound Ras in serum-starved Chang liver cells. Ras protein was immunoprecipitated with Ras-specific monoclonal antibody Y13-259 (26) from cells labeled with [^{32}P]orthophosphate that had been infected with Ad-CMV-X or Ad-CMV-X$_0$ for 3 hr. Nucleotides were eluted, separated by TLC, and visualized by autoradiography; spots were removed from plates and quantified by scintillation counting. Positions of GDP and GTP standards are indicated. (*B*) Requirement for Ras activity in HBx induction of AP-1 DNA-binding activity in recombinant Ad-X-infected cells. Chang liver cells were transfected with the Ras dominant negative (d.n.) mutant Asn-17 (27) for 48 hr and then infected with Ad-CMV-X or Ad-CMV-X$_0$ for 3 hr. Duplicate plates of cells were pretreated with 5 nM calphostin C (CalC) for 3 hr prior to infection. Cells were harvested, nuclear extracts were prepared, and equal amounts of protein 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 by incubation of 3 μg of nuclear extract protein, labeled oligonucleotide, and 1 μg of poly[d(I-C)] for 30 min at 23°C. Protein–DNA complexes were resolved by electrophoresis in 4% polyacrylamide gels and visualized by autoradiography. (*C*) Requirement for Ras activity in HBx induction of AP-1 in transfected cells. Serum-starved Chang liver cells were transfected with plasmids expressing HBx or HBx$_0$, with and without a plasmid expressing the Ras Asn-17 dominant negative (d.n.) mutant. Cells were harvested and nuclear extracts were assayed for AP-1 DNA-binding activity as described for *B*. In competition experiments, a 100-fold molar excess of unlabeled ("cold") oligonucleotide was added to the binding reaction mixture.

addition of 40 μl 2× Laemmli sample buffer, and proteins were denatured and subjected to SDS/18% PAGE and autoradiography. Antibodies to MAP kinases were obtained from Upstate Biotechnology (Lake Placid, NY).

[^{3}H]Thymidine Incorporation. Chang liver cells were serum starved for 24 hr and then either uninfected or infected with Ad-CMV-X or Ad-CMV-X_0 viruses at 25 plaque-forming units per cell. Normal medium with serum was added. [^{3}H]Thymidine (5 μCi/ml) was added for 30 min after infection at time 0 (30 min after infection), 3 hr, and 24 hr. Equal numbers of cells were rinsed twice with ice-cold PBS and lysed in 40 mM Tris·HCl, pH 8.0/10 mM EDTA/0.2% SDS. Samples were precipitated with trichloroacetic acid and incubated in 0.3 M NaOH for 30 min at 60°C before scintillation counting. Data shown are the average of three independent experiments.

RESULTS AND DISCUSSION

Ras is a member of a family of low molecular weight GTP-binding proteins that play key roles in the regulation of proliferation and differentiation of all eukaryotic cells by initiating a cascade of threonine and serine phosphorylation events through Raf and MAP kinases (32, 33). Ras proteins bind guanine nucleotides with high affinity, possess an intrinsic guanosine triphosphatase (GTPase) activity that cycles between an active GTP-bound state and an inactive GDP-bound state, and link receptor and nonreceptor signaling pathways (34). To study the effect of HBx on the activation of Ras, we assayed for the conversion of the inactive, GDP-bound form of Ras into the active, GTP-bound form (Fig. 1*A*). The HBx gene was introduced into Chang liver cells via a replication-defective recombinant Ad vector that expresses the HBx gene in place of regions E1a and E1b (Ad-CMV-X). A control virus (Ad-CMV-X_0) contains a mutated HBx open reading frame lacking all AUG codons (HBx_0) and therefore fails to synthesize HBx protein. Studies from our lab have demonstrated that recombinant Ad-X vectors are genetically silent other than for expression of HBx protein (ref. 12; J.B. *et al.*, unpublished work). The use of a recombinant Ad vector permitted rapid introduction of HBx into cells and kinetic analysis of HBx induction of cellular signaling pathways. Expression of HBx stimulated formation of Ras–GTP (active) complexes by 1.4-fold (64%) over that in unstimulated (basal) control cells (45%) or those containing HBx_0 virus (43%) (Fig. 1*A*). A kinetic analysis showed that HBx activation of Ras plateaued by 3 hr after its introduction (data not shown), which correlates in time with

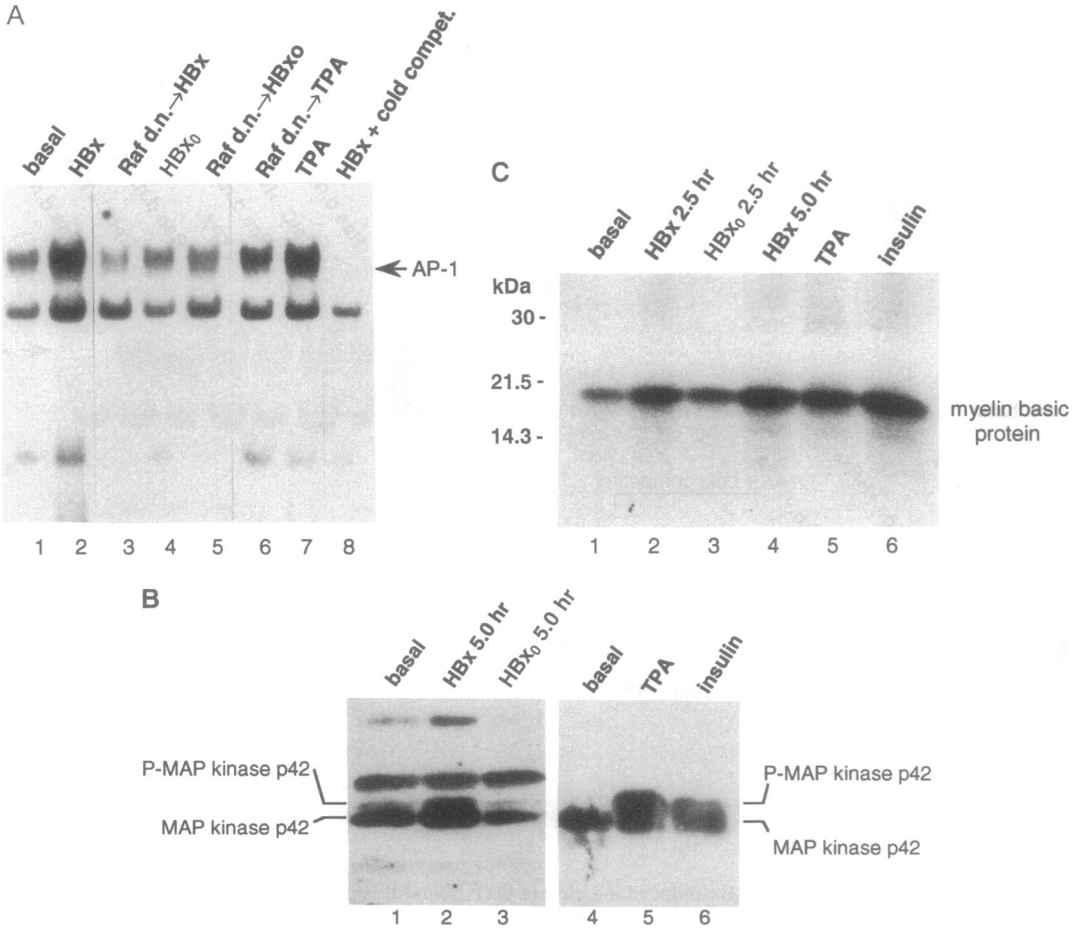

FIG. 2. Effect of HBx on Raf and MAP kinase activities. (*A*) Effect of Raf dominant negative mutant C4 on HBx induction of AP-1 DNA-binding activity. Serum-starved Chang liver cells were transfected for 48 hr with plasmids encoding Raf mutant C4 and then infected with Ad-CMV-X or Ad-CMV-X_0. Nuclear extracts were prepared 3 hr later and equal amounts of protein were used to measure AP-1 DNA-binding activity by band-shift assay as described in the legend to Fig. 1. cold compet., unlabeled oligonucleotide competitor. (*B*) HBx-induced phosphorylation of MAP kinase. Cells were infected with Ad-CMV-X or Ad-CMV-X_0, or stimulated with insulin (10 μg/ml) or TPA (50 ng/ml), or left untreated. Whole cell extracts were resolved by SDS/PAGE, and proteins transferred to nitrocellulose for immunoblot analysis with a polyclonal antibody to pp42/44 (lanes 1–3) or pp42 (lanes 4–6) MAP kinase. The slower migrating form of MAP kinase corresponds to activated (phosphorylated, P) kinase. (*C*) MAP kinase activity induced by HBx. Cells were infected with Ad-CMV-X or Ad-CMV-X_0, or stimulated with insulin or TPA, or left untreated as described above. Cell lysates were immunoprecipitated with antibody to MAP kinase, and washed immunoprecipitates were incubated with purified myelin basic protein and [γ-^{32}P]ATP at 30°C for 30 min. Kinase reactions were stopped by the addition of Laemmli sample buffer and analyzed by SDS/PAGE and autoradiography.

maximal induction of AP-1 DNA-binding activity in these cells (J.B. *et al.*, unpublished work). Levels of Ras–GTP induced at 3 hr by HBx were also comparable to maximal stimulation with phorbol ester (TPA) for 5 min (Fig. 1*A*) and levels of stimulation typically observed by others (e.g., refs. 27 and 34).

These data suggested that the ability of HBx to activate Ras might account for its transcriptional transactivation activities. This was directly addressed by determining whether HBx induction of AP-1 DNA-binding activity is prevented by transfection of Chang cells with a Ras dominant negative mutant (Asn-17) that blocks formation of (active) Ras–GTP (27). Chang cells were found to be very efficiently transfected by plasmid DNAs (average 80% efficiency), determined by *in situ* staining of cells for expression of the β-galactosidase gene expressed from a cotransfected plasmid (data not shown) that is under the control of the Ad major late promoter and is not responsive to HBx (12). As reported previously, HBx induces strong activation of AP-1 DNA-binding activity in a variety of cell lines (refs. 6 and 7; J.B. *et al.*, unpublished work). AP-1 binding complexes were examined by DNA band-shift assay (Fig. 1*B*). Transfection of cells with the Ras dominant negative mutant fully blocked subsequent induction of AP-1 by HBx delivered from a viral vector or induction by TPA. As expected, mutant HBx_0 failed to induce AP-1/DNA complexes (Fig. 1*B*), consistent with its failure to activate Ras. When HBx or HBx_0 genes were introduced into cells by transfection of plasmids rather than by infection with recombinant viruses, cotransfection with the Ras dominant negative inhibitor also prevented activation of AP-1 DNA-binding activity by HBx (Fig. 1*C*). These data therefore confirm that the activation of Ras and induction of AP-1 observed with the recombinant Ad-CMV-X vector are mediated by the HBx protein and not by unexpected gene activities from the viral vector. Moreover, since the HBx and Ras dominant negative mutant genes are controlled by the same promoter, we can exclude the possibility that the dominant negative Ras simply suppressed HBx synthesis. HBx induction of AP-1 was unaffected by treatment of cells with calphostin C, a highly potent and specific inhibitor of Ca^{2+}/diacylglycerol-dependent PKCs (35), at a concentration (5 nM) that largely blocked induction of AP-1 complexes by TPA (Fig. 1*B*). This result agrees with previous reports from our lab and others demonstrating that in most cells HBx activates transcription factor AP-1 without a requirement for PKC α, β, or γ (refs. 7, 12, 24, and 25; J.B. *et al.*, unpublished work). Thus HBx activation of Ras is required for transactivation of AP-1.

To map the downstream events transduced by HBx-activated Ras, the activity of Raf was next examined. The Raf family of serine/threonine kinases function downstream of Ras in mammalian cells (27), as shown by the ability of a dominant negative mutant of Raf kinase (Raf C4) to block Ras functions (36). Cells were transfected with the Raf C4 mutant and then infected with HBx or HBx_0 recombinant Ad vectors. The Raf C4 dominant negative mutant blocked HBx induction of AP-1 DNA-binding activity to levels of uninduced (basal) cells (Fig. 2*A*). The Raf C4 mutant also largely prevented TPA induction of AP-1 DNA-binding activity (Fig. 2*A*). The inability of TPA to block induction of AP-1 observed in Figs. 1 and 2 reflects the failure to transfect >80% of cells with Ras or Raf dominant negative plasmids. These results therefore illustrate the requirement for HBx-activated Raf in induction of AP-1 DNA-binding activity. Activation of Raf requires phosphorylation on serine residues, which causes a characteristic retarded mobility in electrophoretic gels (37). A slower migrating, hyperphosphorylated form of Raf was evident by immunoblot assay and was maximally induced by 3 hr after introduction of HBx, but was not observed with HBx_0 (data not shown). These results confirm that activated Raf is linked in a signaling cascade induced by HBx and leading to transcriptional transactivation.

In mammalian cells Raf transduces signals to MAP kinase by first phosphorylating and activating MAP kinase kinase (Mek) (38). Activated Mek activates MAP kinase by tyrosine and threonine phosphorylation (39, 40). We next determined whether HBx stimulates signaling downstream of Raf to MAP kinase. Lysates from control and Ad-CMV-X (HBx)-infected cells were resolved by SDS/PAGE, and the separated proteins were transferred to nitrocellulose and probed with a polyclonal antibody to the pp42 and pp44 forms of MAP kinase (Fig. 2*B*, lanes 1–3) or only the pp42 form (lanes 4–6). Between 2.5 and 5 hr after introduction of HBx, corresponding to maximal activation of AP-1 DNA-binding complexes, a significant proportion of an electrophoretically slower form of MAP kinase was observed only in cells containing HBx or stimulated with insulin for 15 min. The electrophoretic shift is characteristic of activated MAP kinase due to enhanced phosphorylation (8). The ratio of phosphorylated to nonphosphorylated p42 MAP kinase averaged 3% for basal and HBx_0 samples, 20% for HBx and insulin induction, and 50% for induction by TPA. Activation of MAP kinase by HBx was independently confirmed by examining the ability of MAP kinase immunoprecipitates to phosphorylate a specific substrate, myelin basic protein. MAP kinase activity was enhanced 10- to 20-fold in lysates from cells containing HBx for 2.5–5 hr or from cells treated with insulin for 15 min, but not in uninduced cells or those containing HBx_0 (Fig. 2*C*). Moreover, MAP kinase immunoprecipitates obtained from cells transfected with the Raf C4 mutant prior to introduction of HBx lacked significant MAP kinase-phosphorylating activity (data not shown). These results therefore link Ras, Raf kinase, and MAP kinase activities in a signaling pathway activated by HBx and suggest that MAP kinase is a downstream effector of HBx transactivation activities.

It was next shown that HBx directly activates the Ras, Raf, MAP kinase cascade and does not induce secretion of autocrine factors that then activate receptor tyrosine kinases coupled to Ras. Conditioned medium obtained from cells expressing HBx for 3 hr failed to induce AP-1 DNA-binding

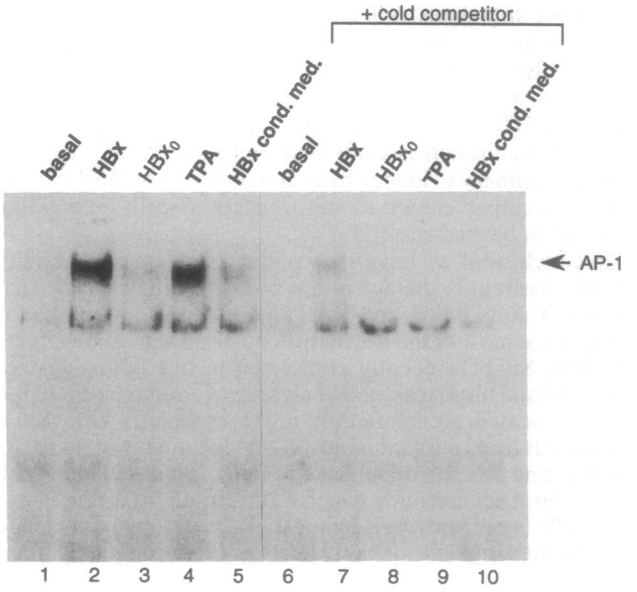

FIG. 3. Intracellular activation of Ras signaling by HBx. Chang liver cells were serum starved and infected by Ad-CMV-X for 3 hr, when maximal activation of Ras and AP-1 activities is induced. Conditioned medium (cond. med.) from these cells was used to culture uninfected serum-starved Chang cells for 3 hr. AP-1 DNA-binding activity in nuclear extract was analyzed by electrophoretic band-shift assay.

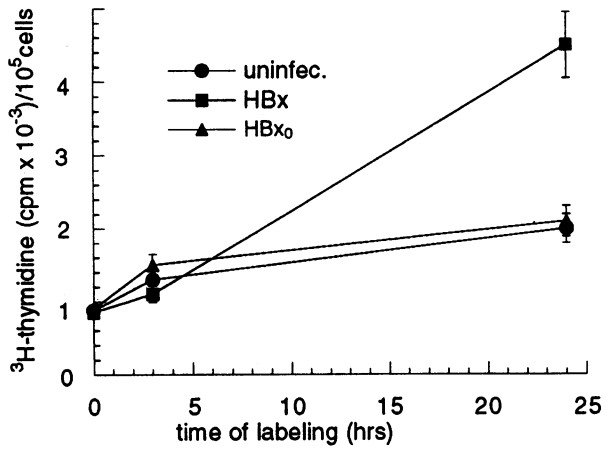

FIG. 4. Induction of cellular DNA synthesis by HBx. Chang cells were serum starved for 24 hr and then infected with recombinant HBx viruses at 25 plaque-forming units per cell, and normal medium with serum was added. Cells were labeled for 30 min with [^{3}H]thymidine at 30 min postinfection (time 0), 3 hr, and 24 hr. Control (uninfected) cells were stimulated with 10% serum for the same times. Cells were washed and lysed, and the amount of [^{3}H]thymidine incorporated into DNA was determined.

activity when used to culture control (unstimulated) cells (Fig. 3). Conditioned medium from HBx-expressing cells also failed to elevate the Ras–GTP level above that of unstimulated control cells (data not shown). Thus, HBx activates Ras intracellularly, which signals to Raf and MAP kinases.

Given that activation of the Ras, Raf, MAP kinase pathway can play a central role in control of cell growth and proliferation, the effect of HBx on stimulation of cell DNA synthesis was investigated. HBx or HBx_0 were introduced into serum-starved cells by viral vectors, and stimulation of cell DNA synthesis was assayed at various times by [^{3}H]thymidine incorporation. By 24 hr, HBx stimulated [^{3}H]thymidine incorporation to 2–2.5 times that of uninfected, serum-stimulated cells (Fig. 4). Expression of HBx_0 had no effect on [^{3}H]thymidine incorporation. These data therefore indicate that HBx stimulates cellular DNA synthesis in Chang cells and thus may promote cell proliferation. These experiments cannot distinguish whether increased [^{3}H]thymidine uptake is due to stimulation of *de novo* DNA synthesis or repair, although HBx induction is only 2-fold higher than that of serum, which is consistent with *de novo* synthesis. Additionally, preliminary evidence has been obtained by flow cytometry, indicating enhanced mitogenesis in cells expressing HBx (unpublished results).

In conclusion, we have shown that a fundamental activity of HBx protein is the activation of a signaling cascade that links Ras, Raf kinase, and MAP kinase and that accounts for many if not most of the transcriptional transactivation effects of HBx. Studies recently completed in our lab using biochemical and ultrastructural analyses have demonstrated that HBx is located predominantly in the cytoplasm and that it carries out transactivation only in that location (M. Doria, R. Lucito, and R.J.S., unpublished work). Thus, although the molecular mechanism by which HBx stimulates formation of Ras–GTP is not known, HBx most likely acts on cytoplasmic factors that regulate or activate Ras–GTP complex formation.

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