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Recruitment of hepatocyte nuclear factor 4 into specific intranuclear compartments depends on tyrosine phosphorylation that affects its DNA-binding and transactivation potential

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ABSTRACT **Hepatocyte nuclear factor 4 (HNF-4) is a prominent member of the family of liver-enriched transcription factors, playing a role in the expression of a large number of liver-specific genes. We report here that HNF-4 is a phosphoprotein and that phosphorylation at tyrosine residue(s) is important for its DNA-binding activity and, consequently, for its transactivation potential both in cell-free systems and in cultured cells. Tyrosine phosphorylation did not affect the transport of HNF-4 from the cytoplasm to the nucleus but had a dramatic effect on its subnuclear localization. HNF-4 was concentrated in distinct nuclear compartments, as evidenced by *in situ* immunofluorescence and electron microscopy. This compartmentalization disappeared when tyrosine phosphorylation was inhibited by genistein. The correlation between the intranuclear distribution of HNF-4 and its ability to activate endogenous target genes demonstrates a phosphorylation signal-dependent pathway in the regulation of transcription factor activity.**

Control of gene expression at the level of transcription is a predominant mechanism for the activation of tissue-specific transcription factors. Hepatocyte nuclear factor 4 (HNF-4) is one such transcription factor with limited tissue distribution which was thought to be regulated mainly at the transcriptional level (1, 2). HNF-4 can activate transcription in several nonhepatic cell lines, indicating that no liver-specific modification is required for its function (3). On the other hand, the apparent contradiction between the molecular mass of HNF-4 predicted from the primary sequence (50.6 kDa) (3) and that determined by gel electrophoresis (54 kDa) suggests that this difference may be due to posttranslational modification(s). Of the many types of posttranslational modifications that might regulate gene expression, most attention has been focused on phosphorylation, which can influence transcription factor activity in many ways (4). Three main levels of regulation have been described: phosphorylation can affect the DNA-binding activity (5–7), the transcriptional activation potential (8, 9), or the translocation of a transcription factor from the cytoplasm into the nucleus (10–13). These possibilities are by no means mutually exclusive, and in principle phosphorylation can be responsible for simultaneous regulation at several distinct levels. With the exception of certain signal transduction proteins (14), all examples of this type of regulation have involved phosphorylation at serine or threonine residues.

Here we demonstrate that the activity of HNF-4 is posttranslationally regulated by tyrosine phosphorylation, providing an example of a non-signal-transduction factor modulated by this modification. The HNF-4 polypeptide contains 12 tyrosine residues scattered throughout the DNA-binding, dimerization, and putative ligand-binding domains (3) which could be potential phosphorylation sites. We did not attempt to determine the exact location of the phosphorylated tyrosine residue(s), but we present evidence that tyrosine phosphorylation of HNF-4 is required for its DNA-binding activity. We show that the transcriptionally active form of HNF-4 is localized in specific subnuclear domains. This intranuclear distribution depends directly or indirectly on tyrosine phosphorylation, suggesting the existence of an additional control mechanism at the level of subnuclear targeting playing a role in transcription regulation.

MATERIALS AND METHODS

Cell Culture and Transfection. Monolayer cultures of HepG2 and COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO) with 10% heat-inactivated fetal bovine serum. Twenty-four hours before transfection the cells were seeded at 50–60% confluency. For chloramphenicol acetyltransferase (CAT) assays the cells were transfected by the calcium phosphate coprecipitation method and assayed as described (15–17). For all other experiments COS-1 cells at 50–60% confluency were incubated with 10 μg (60-mm dishes) or 2.5 μg (coverslips) of DNA in DMEM/10 mM Hepes, pH 7.2/0.025% DEAE-dextran for 1.5 hr at 37°C. The DNA was removed, and the cells were treated with 0.1 mM chloroquine in DMEM/10% fetal bovine serum for 5 hr. The cells were washed with DMEM/10 mM Hepes, pH 7.2, and grown in DMEM/10% fetal bovine serum for 48 hr before harvest.

Metabolic Labeling and Protein Analysis. Monolayer cultures of HepG2 and transfected COS-1 cells were incubated with [^{32}P]orthophosphate (1 mCi/ml; 1 mCi = 37 MBq) or [^{35}S]methionine (0.5 mCi/ml) for 2 hr in phosphate-free or methionine-free DMEM with or without genistein (Upstate Biotechnology) at 15 μg/ml. The cells were washed with phosphate-buffered saline (PBS) and lysed in 50 mM Tris, pH 7.5/150 mM NaCl/1 mM EDTA/0.5% Nonidet P-40/10% glycerol/1 mM Na_3VO_4/1 mM NaF/0.1 mM phenylmethanesulfonyl fluoride with aprotinin at 10 μg/ml. After a preclearing step with protein A-Sepharose (Pharmacia), HNF-4 was immunoprecipitated with anti-HNF-4 (0.5 μl per sample), separated by SDS/10% PAGE, and visualized by autoradiography. In parallel, cell lysates from unlabeled cultures were processed as above, and after SDS/PAGE, proteins were electroblotted to nitrocellulose membranes (Schleicher & Schuell) and probed with 0.5 μg/ml horseradish peroxidase-conjugated anti-phosphotyrosine (PY20; ICN). Antibody was detected by enhanced chemiluminescence (ECL; Amersham). Nuclear extracts were prepared and electrophoretic mobility-shift assays were performed as described (15, 16).

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Abbreviations: CAT, chloramphenicol acetyltransferase; HNF, hepatocyte nuclear factor.
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Immunolocalization Assays. For indirect immunofluorescence assays, cells on coverslips were washed twice with PBS and fixed for 10 min at −20°C with methanol. After fixation the coverslips were washed once with PBS and incubated with PBS containing 1% bovine serum albumin (BSA/PBS) for 30 min at room temperature. Antibodies were diluted 1:200 in BSA/PBS and applied to the cells for 45 min. After washing with BSA/PBS for 15 min the cells were incubated with diluted (1:100) fluorescein isothiocyanate-conjugated anti-rabbit or anti-mouse secondary antibodies to IgG (Pierce) for 30 min at room temperature. The coverslips were washed with BSA/PBS for 15 min, rinsed in water, mounted on glass slides with Mowiol (Polyscience), and observed in a Leitz Dialux 20 EB microscope equipped with epifluorescence optics.

For electron microscopy, pellets from 5×10^7 untreated and genistein-treated HepG2 cells were fixed in 4% formaldehyde/0.5% glutaraldehyde for 1 hr and embedded in LR White resin (Polyscience) for 24 hr at 50°C without accelerator. Thin sections (≈50 nm) were deposited on copper grids and incubated overnight at 4°C with anti-HNF-4 diluted at 1:1000 in BSA/PBS. After extensive washing, the samples were stained with diluted (1:10) gold-conjugated goat anti-rabbit secondary antibodies (particle size: 10 nm or 20 nm) at room temperature for 1 hr. After washing, the samples were stained with 1% uranyl acetate for 10 min and observed in a JEOL 100C electron microscope operated at 100 kV.

RESULTS AND DISCUSSION

To examine whether HNF-4 is phosphorylated, lysates from [^{32}P]orthophosphate-labeled HepG2 and HNF-4-transfected COS-1 cells were immunoprecipitated with HNF-4-specific antiserum (3). A 54-kDa labeled protein band was readily detected by this antibody, indicating that both endogenous HNF-4 (HepG2) and transiently expressed HNF-4 (COS-1) were phosphorylated (Fig. 1 *A* and *B*). When the cells were treated with genistein, a specific protein-tyrosine kinase inhibitor (18), a ^{32}P-labeled protein band with decreased intensity and slightly faster mobility was observed, suggesting that at least in part HNF-4 may be phosphorylated at tyrosine residue(s) (Fig. 1 *A* and *B*). To test this hypothesis, HNF-4 was immunoprecipitated from extracts of HepG2 and transfected COS-1 cells that had been treated with genistein or left untreated, and Western blots of the immunoprecipitates were probed with a monoclonal antibody to phosphotyrosine. A 54-kDa tyrosine-phosphorylated protein band corresponding to HNF-4 was observed in the extracts from the untreated cells but not in the extracts from the genistein-treated cells (Fig. 1*C*). Metabolic labeling with [^{35}S]methionine showed that genistein did not influence the expression and/or intracellular stability of HNF-4 (Fig. 1 *A* and *B*), suggesting that the decrease in ^{32}P and phosphotyrosine signal was solely due to selective inhibition of tyrosine phosphorylation.

To evaluate the role of tyrosine phosphorylation in the modulation of HNF-4 activity, we first examined whether its DNA-binding potential was affected. Nuclear extracts from HepG2 and HNF-4-transfected COS-1 cells were prepared and analyzed in electrophoretic mobility-shift experiments using a specific HNF-4 binding site as a probe (site A; refs. 15 and 16). Nuclear extracts from genistein-treated cells had a very low binding activity when compared with extracts from untreated cells (Fig. 2*A*), suggesting that tyrosine phosphorylation is required for DNA binding. This result was not due to a toxic effect(s) of the drug, since removal of genistein from the culture restored HNF-4 binding activity within 24 hr (Fig. 2*A*). *In vitro* evidence for the direct involvement of tyrosine phosphorylation in DNA binding was obtained by treatment of nuclear extracts with purified protein-tyrosine-phosphatase 1B. This treatment reduced the DNA-binding activity of HNF-4, and the effect was largely reversed by inclusion of the specific protein-tyrosine-phosphatase inhibitor Na_3VO_4 in the binding reaction mixture (Fig. 2*B*). The presence of phosphotyrosine in the DNA-binding form of HNF-4 was further confirmed when incubation with anti-phosphotyrosine antibodies specifically interfered with the formation of the HNF-4/DNA complex (Fig. 2*C*). Since DNA binding is a prerequisite step for transcription activation by HNF-4, we examined how tyrosine phosphorylation affects its activity in living cells. HepG2 and COS-1 cells were transiently transfected with a construct containing four copies of HNF-4 binding site A fused to the −85-nt thymidine kinase minimal promoter (16). Treatment of the cells with genistein decreased transcription activation by the endogenous (HepG2) and the expression vector-derived (COS-1) HNF-4 to 20% and 7% of control (Fig. 3 *A* and *B*). This inhibition was reversed upon removal of the drug, arguing against toxicity as an explanation. In addition, genistein treatment did not significantly influence transcription driven by the Rous sarcoma virus promoter, demonstrating that the cellular transcription apparatus was fully active under our experimental conditions (Fig. 3 *A* and *B*). We also examined the activation of the endogenous HNF-1 gene, whose transcription absolutely depends on HNF-4 (19, 20). COS-1 cells, which normally do not express either HNF-1 or HNF-4, were transfected with pCB-HNF-4 and the activation of the endogenous HNF-1 gene was analyzed in mobility-shift experiments using the high-affinity HNF-1 binding site AlbPE (15, 21) as a probe. HNF-4 induced the formation of a specific DNA/protein complex, which was partially inhibited and partially supershifted by the inclusion of an HNF-1-specific antibody in the binding reaction mixture (Fig. 2*D*). Treatment of the cells with genistein inhibited the activation of this DNA-binding factor. Similar inhibition was observed in HepG2 cells which was reversible upon removal of the drug (Fig. 2*E*). Since the DNA-binding potential of expression vector-derived HNF-1 was not affected by this treatment (Fig. 2*E*), we ascribe this inhibition to the reduced rate of endog-

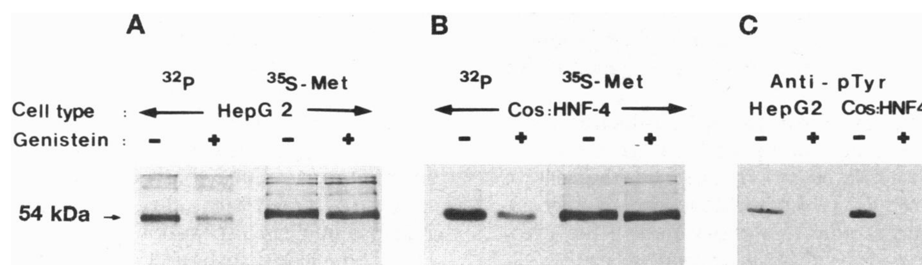

FIG. 1. (*A* and *B*) Tyrosine phosphorylation of HNF-4. HepG2 (*A*) or pCB-HNF-4-transfected COS-1 (*B*) cells were incubated for 2 hr with [^{32}P]orthophosphate or [^{35}S]methionine in the presence (+) or absence (−) of genistein (15 μg/ml). Genistein was added to the culture medium 1 hr before the ^{32}P labeling and 48 hr before the ^{35}S labeling. Cell lysates were subjected to immunoprecipitation with anti-HNF-4 polyclonal antibodies. The immunoprecipitates were separated by SDS/PAGE and visualized by autoradiography. (*C*) Lysates from genistein-treated (+) or untreated (−) HepG2 and pCB-HNF-4-transfected COS-1 cells were immunoprecipitated with anti-HNF-4. After SDS/PAGE, proteins were electroblotted and probed with horseradish peroxidase conjugated anti-phosphotyrosine monoclonal antibody PY20 (anti-pTyr).

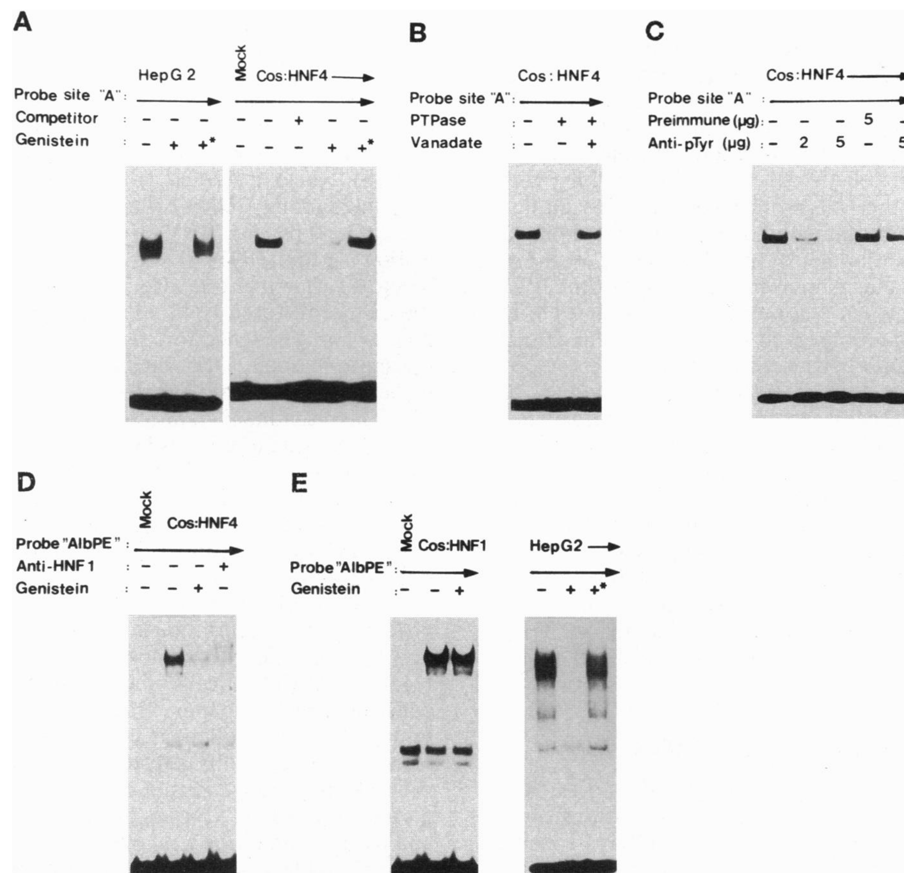

FIG. 2. (*A–C*) DNA-binding activity of HNF-4 is modulated by tyrosine phosphorylation. (*A*) Nuclear extracts from HepG2 and pCB-HNF-4-transfected COS-1 cells were analyzed in mobility-shift experiments using the high-affinity HNF-4 binding site (site A) probe (15, 16). Cells were treated with genistein (15 μg/ml) for 48 hr (+), washed repeatedly with complete medium, and incubated for an additional 24 hr (+*) before extract preparation. (*B*) Nuclear extracts from pCB-HNF-4-transfected COS-1 cells were incubated with 10 units of purified protein-tyrosine-phosphatase 1B (PTPase; Upstate Biotechnology) for 10 min at 30°C in the presence or absence of 1 mM Na_3VO_4 (sodium vanadate) before initiation of the binding reaction. (*C*) Nuclear extracts from pCB-HNF-4-transfected COS-1 cells were preincubated in ice with 2 or 5 μg of polyclonal antibodies to phosphotyrosine (4G10; Upstate Biotechnology) or 5 μg of preimmune antiserum for 3 hr before the binding reaction. In the lane marked with an asterisk the phosphotyrosine antibodies were incubated with 10 mM phosphotyrosine-agarose for 1 hr before incubation with the nuclear extract. (*D* and *E*) Activation of the endogenous HNF-1 in COS-1 cells. COS-1 cells were transfected with pCB-HNF-4 (*D*) or pCB-HNF-1 (*E*) and incubated in the presence (+) or absence (−) of genistein (15 μg/ml). Nuclear extracts were prepared 48 hr after transfection and analyzed in mobility-shift experiments using the high-affinity HNF-1 binding site AlbPE as a probe. In the rightmost lane of *D*, 1 μl of diluted (1:6) HNF-1-specific antiserum was included in the binding reaction. Mobility-shift assays using the AlbPE probe were also performed with extracts from HepG2 cells treated as described in *A*.

enous HNF-1 expression caused by the inactivation of HNF-4. The ability of HNF-4 to activate the endogenous HNF-1 gene was further confirmed by transient transfection experiments using the 3xAlbPE-AdML-CAT (15) reporter. Transfection of COS-1 cells with HNF-4 could activate this chimeric promoter, whose activity is dependent on transcriptionally active HNF-1. As expected, treatment of the cells with genistein resulted in reversible loss of activation (Fig. 3*C*). Taken together, the above results demonstrate that tyrosine phosphorylation of HNF-4 is required for DNA binding and consequently for transactivation.

Recent studies on the interferon signal transduction pathways led to the discovery of a family of latent transcription factors (STAT family) activated by tyrosine phosphorylation (14). Upon cytokine- or growth factor-dependent induction, STAT proteins become rapidly phosphorylated in the cytoplasm and subsequently translocate to the nucleus (13). Although phosphorylation of HNF-4 is not dependent on several of the known extracellular signaling proteins (E.K., unpublished observations), it was of interest to determine whether tyrosine phosphorylation affects the transport of HNF-4 from the cytoplasm into the nucleus. To this end we compared the intracellular distribution of HNF-4 in untreated and genistein-treated HepG2 and transfected COS-1 cells by indirect immunofluorescence analysis using polyclonal antiserum specific for HNF-4. Predominantly nuclear staining was observed in both cases, indicating that tyrosine phosphorylation does not play a role in the nuclear translocation of HNF-4 (Fig. 4*A*, *a–d*). However, examination of the nuclear signal at high magnification revealed a striking difference in the subnuclear topology of HNF-4 between the untreated and genistein-treated cells. The HNF-4 signal accumulated in distinct nuclear compartments in both HepG2 and transfected COS-1 cells (Fig. 4*A*, *e* and *h*). Five to 15 such loci containing the majority of immunoreactive HNF-4 were observed in the nuclei of both cell types, suggesting that their number does not depend on the different intracellular levels of HNF-4. In contrast, when the cells were treated with genistein this pattern disappeared and a diffuse nuclear distribution was observed (Fig. 4*A*, *f* and *i*). The subnuclear distribution of HNF-4 was further characterized by immunoelectron microscopy. Thin sections of untreated and genistein-treated HepG2 cells were immunolabeled by anti-HNF-4 followed by colloidal gold-conjugated secondary antibodies. One to three immunopositive regions, containing assembled groups of 20–40 immunogold particles, were observed in most sections of untreated cells, while no

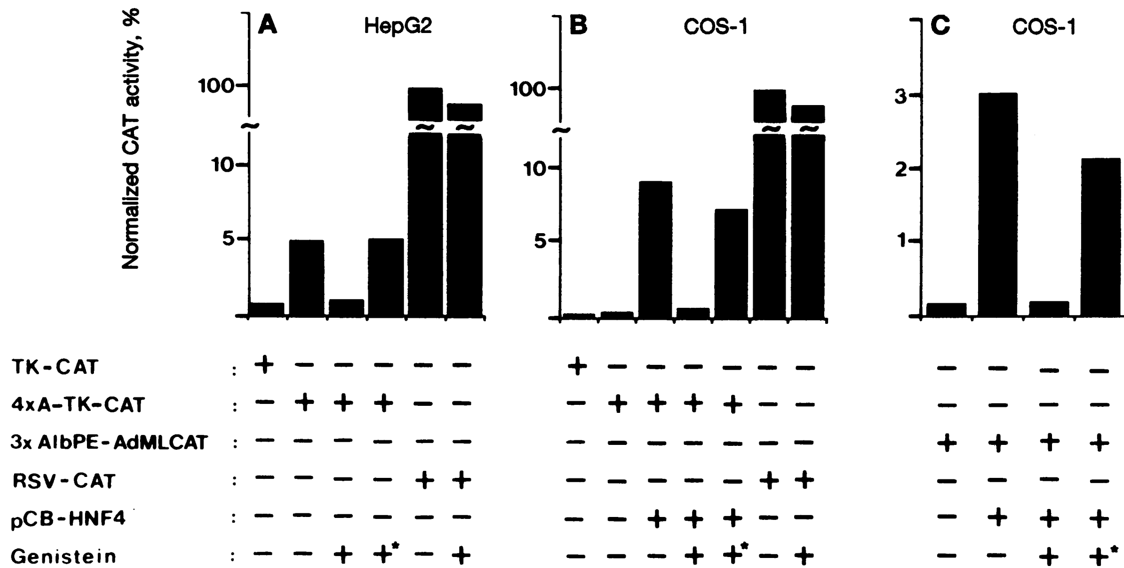

FIG. 3. Analysis of HNF-4-dependent transcriptional activation. HepG2 and COS-1 cells were transfected (calcium phosphate method) with 2 μg of CAT reporter constructs in which transcription is controlled by the minimal thymidine kinase promoter (TK-CAT), four copies of the specific HNF-4 binding site A fused to the TK promoter (4xA-TK-CAT; ref. 16), three copies of the high-affinity HNF-1 site AlbPE fused to the adenovirus major late promoter (3xAlbPE-AdML-CAT; ref. 15) or the Rous sarcoma virus promoter (RSV-CAT; ref. 15). Where indicated, COS-1 cells also received 2 μg of pCB-HNF-4 expression vector. After glycerol shock the cells were cultured in the presence (+) or absence (−) of genistein (15 μg/ml) for 40 hr. In some cultures the medium was replaced at this time and the cells were cultured for an additional 24 hr (+*). Bars represent mean values of normalized CAT activities from at least four independent experiments, expressed as a percentage of RSV-CAT activity (not shown in *C*).

such accumulations were evident in cells treated with genistein (Fig. 5). The cytoplasm and nucleoli did not show any immunoreactivity. These findings suggest that the distinct immunoreactive regions observed in the *in situ* fluorescence experiments correspond to specific assembly of HNF-4 molecules into discrete nuclear subdomains.

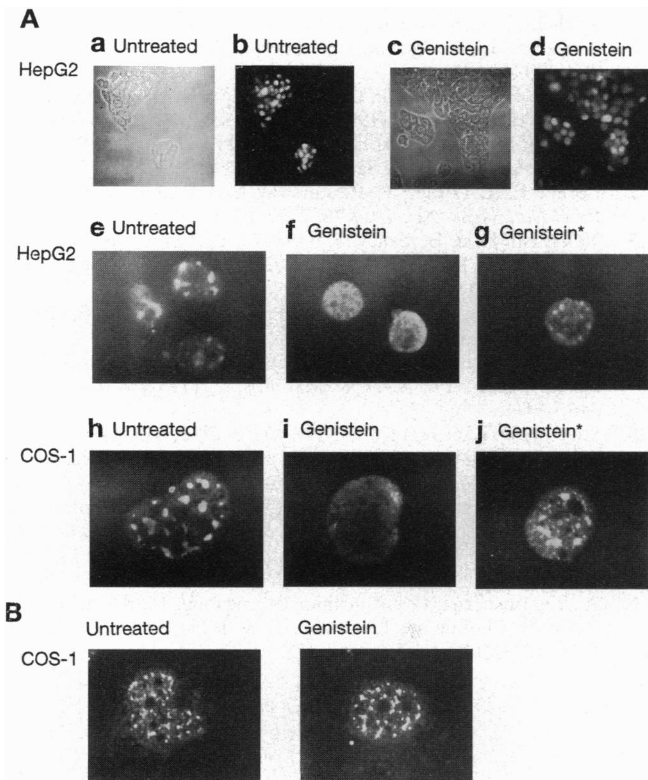

FIG. 4. Immunolocalization of HNF-4 in HepG2 and COS-1 cells. COS-1 cells were plated on coverslips, transfected with pCB-HNF-4, and then left untreated or treated with genistein (15 μg/ml) for 48 hr. Then some cells were extensively washed with complete medium and incubated for an additional 24 hr (genistein*). Coverslips containing HepG2 cells were treated similarly. Immunostaining of the cells was performed with anti-HNF-4 (*A*) or anti-Sm monoclonal antibody Y12 (*B*). In *A*, *a* and *c* show corresponding phase-contrast images of *b* and *d*, respectively. (*A a–d*, ×60; *A e–j* and *B*, ×650.)

Three independent observations argue against the possibility that the diffuse nuclear distribution of HNF-4 in the genistein-treated cells may be attributed to some sort of changes in the nuclear architecture caused by the drug. First, the punctate pattern of HNF-4 distribution was regenerated within 24 hr upon removal of the drug from the culture medium (Fig. 4*A*, *g* and *j*). Second, the characteristic speckled nuclear pattern obtained with the Y12 antibody, which recog-

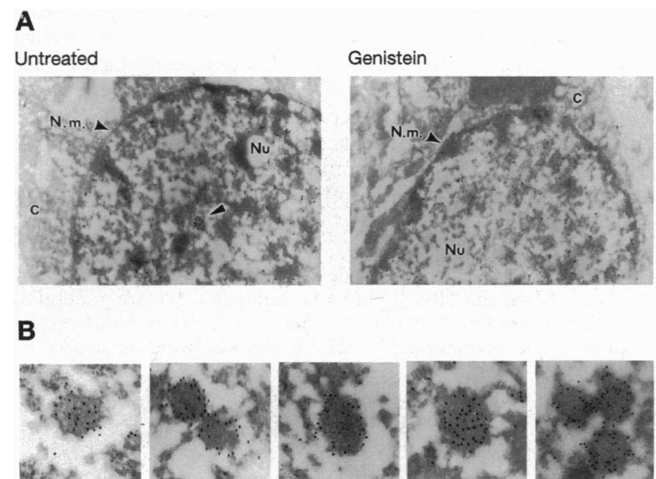

FIG. 5. Ultrastructural localization of HNF-4 in HepG2 cells. Thin sections of untreated and genistein-treated HepG2 cells were immunostained with anti-HNF-4 and examined by electron microscopy. (*A*) Low magnification of untreated and genistein-treated HepG2 cells. Unlabeled arrowhead in *A* indicates clustered HNF-4 nuclear distribution seen only in untreated cells. C, cytoplasm; N.m., nuclear membrane; Nu, nucleus. (×6300.) (*B*) Higher magnification of five examples of HNF-4-containing nuclear clusters seen in untreated HepG2 cells. (×28,000.)

nizes small nuclear ribonucleoproteins involved in pre-mRNA splicing (22), was not changed by genistein treatment (Fig. 4*B*). Third, electron microscopy showed no obvious morphological alterations in the nuclear structure in the genistein-treated cells. Therefore the effect of genistein on HNF-4 localization is due to the inhibition of tyrosine phosphorylation of either HNF-4 itself or a nuclear matrix protein responsible for directing HNF-4 to the specific subnuclear sites. On the basis of the available data, neither explanation can be excluded. Whichever mechanism applies, our results strongly suggest that tyrosine phosphorylation either directly or indirectly plays a regulatory role in the subnuclear distribution of HNF-4.

Several nuclear activities, such as DNA and RNA synthesis and RNA splicing, have been localized in discrete subnuclear domains rather than diffusely distributed throughout the nucleoplasm (23–26). The best studied factors are the components of the splicing machinery, which have been shown to be associated with at least three distinct nuclear structures: perichromatin fibrils, which correspond to the *in situ* forms of nascent heterogeneous nuclear RNA transcripts; interchromatin granules; and coiled bodies (27, 28). PML, a nuclear protein involved in the t(15;17) chromosomal translocation in acute promyelocytic leukemia, is also compartmentalized in the nucleus of acute promyelocytic leukemia cells expressing a PML–retinoic acid receptor α fusion protein, in multiple small nuclear clusters (29–31). Upon administration of retinoic acid, PML relocates into nuclear structures of unidentified function termed nuclear bodies (31). Although the HNF-4-immunoreactive regions observed by electron microscopy resemble the pattern obtained with PML in nuclear bodies, at present we are unable to relate them unequivocally to these structures. However, we speculate that they may correspond to sites where HNF-4 is actively engaged in the transcriptional activation of its target genes. This speculation seems to be valid in light of the positive correlation between the ability of HNF-4 to activate the endogenous HNF-1 gene (Figs. 2 and 3) and the subnuclear localization of its functionally active and inactive forms (Figs. 4 and 5).

Gene activation in general requires the simultaneous presence of several transcription factors which interact with a given array of cis-acting elements on promoter regions. Since most of these factors are present in extremely low amounts in the cells, their random distribution in the nucleoplasm would result in insufficient local concentrations around the regulatory regions for the formation of transcriptionally active complexes. The recruitment of a transcription factor into discrete nuclear locations described in this paper may serve as a mechanism to achieve local concentrations above the threshold level required for gene activation. At least for HNF-4, reaching these specific assembly sites depends on tyrosine phosphorylation of either HNF-4 itself or a carrier nuclear matrix protein.

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