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Similarities in transthyretin gene expression and differences in transcription factors: Liver and yolk sac compared to choroid plexus

(differentiation/cell specificity)

ROBERT H. COSTA*, TERRY A. VAN DYKE†, CHEN YAN†, FRANK KUO‡, AND JAMES E. DARNELL, JR.‡§

*Department of Biochemistry, University of Illinois College of Medicine, 1853 West Polk Street, Chicago, IL 60612; †Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260; and ‡Laboratory of Molecular Cell Biology, Rockefeller University, New York, NY 10021

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ABSTRACT **The serum thyroxine-binding protein, transthyretin (TTR), is made by hepatocytes and by choroid plexus epithelium in adults and by yolk sac cells in embryogenesis. Four hepatocyte nuclear factors (HNF-1, -3, and -4 and C/EBP) that are present in liver but not in most other adult tissues bind DNA sites in the TTR gene that are sufficient to direct transgenic expression. Three of these proteins were also found in yolk sac cells, which also express the transgene. A limited transgenic construct is not active in the choroid plexus and a TTR-producing choroid plexus tumor lacks three of the liver-enriched DNA-binding proteins. We conclude that cell-specific expression of TTR is regulated at least in part by the differential cellular distribution of positive-acting transcription factors.**

The expression of a specific set of genes in hepatocytes offers a good opportunity to define a group of transcription factors responsible for cell-specific gene expression (1, 2). The yolk sac, an embryonic tissue of endodermal origin, as is the fetal liver, also produces many secretory proteins that adult hepatocytes produce (3–5). In addition, a restricted few of the genes expressed in a "hepatocyte-specific" manner are expressed at high levels in occasional other cell types. For example, the transthyretin (TTR) gene is expressed at high levels in hepatocytes and choroid plexus epithelium in adults and in liver, visceral yolk sac cells, and choroid plexus primordia in the embryo but is absent in almost all other adult or fetal cell types (4, 5).

Here we investigate the presence of yolk sac, fetal liver, and choroid plexus cells of transcription factors identified in liver that act in TTR expression, and correlate the presence of factors with endogenous and transgenic expression from the TTR promoter.

MATERIALS AND METHODS

Nuclear Protein Extracts, Oligonucleotides, and Gel Retardation Assays. Protein extracts were made from nuclei isolated from adult or fetal (17-day embryos) rat tissues as described (6). Labeled double-stranded oligodeoxynucleotides (6), each containing a different specific binding site for a hepatocyte nuclear factor (HNF), were used in gel retardation assays (7, 8) to detect the presence of HNF protein–DNA complexes in nuclear extracts. The labeled oligonucleotides used were the HNF-1 probe [rat β-fibrinogen gene, base pairs −90 to −61 relative to the transcription start site (9)], the HNF-3 probe [mouse TTR, −111 to −85 (6)], the HNF-4 probe [mouse TTR, −151 to −130 (6)], the apolipoprotein factor 1 (APF-1) probe [rat apolipoprotein C-III, −91 to −66 (10)], and the activating protein 1 [AP-1/c-Jun (11)] probe [α_1-antitrypsin, −297 to −272 (oligo B in ref. 12)]. After incubation of labeled oligonucleotides with nuclear extracts, protein–DNA complexes were separated from unbound DNA by electrophoresis in nondenaturing acrylamide gels under conditions described previously (6). Competitions were performed by inclusion of a 200-fold molar excess of unlabeled oligonucleotide in the binding reaction unless indicated otherwise.

Transgenic Mice and RNA Analysis. Transgenic mice were generated previously with different portions of TTR upstream sequence driving the expression of a TTR reporter minigene (13). These included minigene constructs with either 3 kilobases (kb) of mouse TTR DNA sequence 5′ to the cap site (designated as TTRA; TA) or 100 nucleotides of TTR distal enhancer (−1.96 to −1.86 kb) fused to the promoter-proximal sequence at −202 (designated as TTRB, TB). Two mice from two different transgenic lines generated from each construct were analyzed for expression in fetal liver and visceral yolk sac. Expression levels were determined by hybridization of total RNA with a labeled antisense RNA probe followed by digestion with RNase T2 (13). Specific transgene expression is distinguished from endogenous expression by protection of a larger RNA fragment from digestion by RNase T2 in the same assay. Transgenic transcription levels from the TTR minigene were expressed relative to endogenous TTR RNA present in each tissue.

RESULTS

By deletion analysis, mutagenesis, and protein–DNA binding assays with purified proteins or nuclear extracts, the TTR gene is known to have at least nine required protein-binding sites and the possible cognate protein for each is known (6, 12, 14, 15). Four of these proteins are enriched in liver extracts: HNF-1, a homeobox-containing protein (16, 17): C/EBP, the original leucine-zipper protein (18, 19), and HNF-3 and -4 (6), two proteins that we have purified (E. Lai, V. Prezioso, E. Smith, O. Litvin, R.H.C., and J.E.D., unpublished work) and for which cDNAs have recently been isolated (F. Sladek, W.-M. Zhong, E. Lai, and J.E.D., unpublished work).

During the course of studying the HNF-4 protein, it was recognized that the TTR sequence to which HNF-4 binds (−141 to −151; TGAACCTTGCC) was similar to a regulatory region of the apolipoprotein C3 gene (−82 to −72; TGACCTTTGCC) that is also expressed mainly in hepatocytes and in intestinal cells (10). Therefore, we used gel retardation (7, 8) to compare the oligonucleotide from the

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Abbreviations: TTR, transthyretin; HNF, hepatocyte nuclear factor; APF, apolipoprotein factor; AP, activator protein.

§To whom reprint requests should be addressed.

TTR promoter that was used originally to identify HNF-4 (nucleotide −151 to −130) with an oligonucleotide from the apolipoprotein C-III gene (APF-1 probe; Fig. 1). The −151 to −130 TTR oligonucleotide has both an HNF-3 and an HNF-4 binding site (6). An unlabeled oligonucleotide with a separate strong HNF-3 binding site [−111 to −85 of the TTR gene (6)] served to distinguish the two sites. Two groups of protein–DNA complexes were formed with the −151 to −130 TTR probe that were eliminated by competition with homologous unlabeled oligonucleotide (6). Excess unlabeled HNF-3 oligonucleotide inhibited formation of the faster-migrating labeled complexes without affecting the slower moving bands that define the original HNF-4 binding site. In contrast, the APF-1 oligonucleotide inhibited the formation of the slower-migrating (HNF-4) complexes without affecting the faster-migrating bands; only a 10-fold molar excess of APF-1 oligonucleotide was required, suggesting a high affinity of the HNF-4 protein for the apolipoprotein gene sequence (Fig. 1). The labeled APF-1 oligonucleotides formed complexes indistinguishable from those formed by a labeled synthetic HNF-4 site (data not shown), indicating that the same or a closely related protein can bind both sites. In subsequent experiments we used either of these sites to detect the HNF-4 protein complex.

HNF Binding Activity Is Present in Fetal Nuclear Extracts. Since many secretory proteins expressed in adult hepatocytes are also expressed in visceral yolk sac and fetal liver (3–5), we prepared nuclear extracts from these three tissues to test for the presence of specific binding proteins by the gel retardation assay (Fig. 2). Each nuclear extract contained proteins that produced retarded complexes (whose formation was inhibited by unlabeled oligonucleotides in each case) with each of a group of labeled oligonucleotides including the HNF-1, HNF-3, and HNF-4 (APF-1) binding sites and the probe recognized by the widely distributed AP-1 binding protein (refs. 8 and 21; Fig. 2). Thus three of the four nuclear factors that are present in hepatocytes and are important in TTR expression in hepatoma cells are also present in fetal liver and visceral yolk sac cells. *In situ* hybridization analysis of 13-day mouse embryos has also detected mRNA for the C/EBP factor in fetal liver (22). However, in those experiments the C/EBP mRNA was not detectable above background in yolk sac cells.

Transgenic Expression. Transgenic mouse lines containing TTR constructs were made earlier (13). Animals bearing constructs containing a TTR minigene with either 3 kb of upstream sequence (transgenic mouse lines TA) or the 100-nucleotide enhancer (−1.96 to −1.86 kb) plus 202 nucleotides upstream of the RNA start site (transgenic mouse lines TB) showed extensive expression of the TTR minigene in hepatocytes [ref. 13 and Fig. 3, lanes A.L. (adult liver) and open bars]. However, only animals with 3 kb of upstream region expressed TTR in the choroid plexus cells as tested by *in situ* hybridization and autoradiography (13). Animals with both types of constructs were then tested for expression of the transgene in yolk sac and fetal liver. An RNase T2 protection

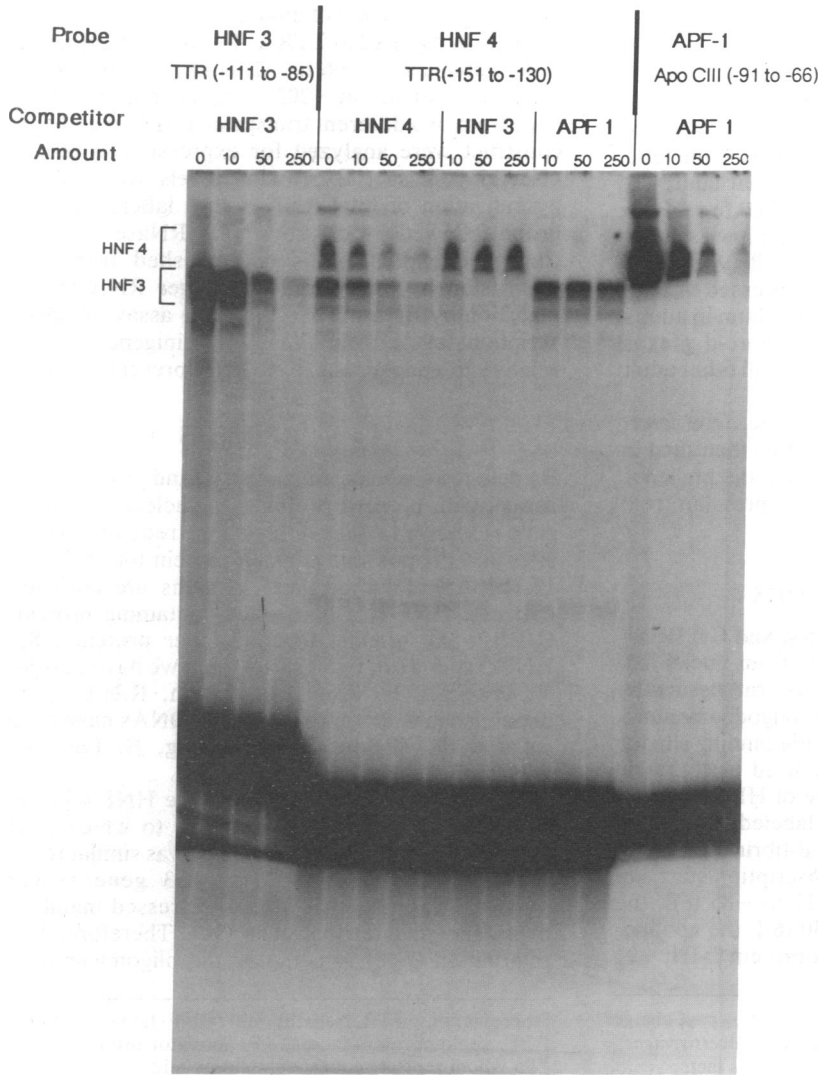

FIG. 1. HNF-4 binds to an apolipoprotein (Apo) C-III sequence. Gel retardation assays (2, 9, 20) were carried out by incubating rat liver nuclear extracts with the indicated labeled double-stranded oligodeoxynucleotide and identifying labeled protein–DNA complexes (brackets) by electrophoresis followed by autoradiography (6). The labeled oligonucleotides were the HNF-3 probe (nucleotides −111 to −85 of the mouse TTR gene), the HNF-4 probe [−151 to −130 of the mouse TTR gene (6)], the APF-1 probe [−91 to −66 of the human apolipoprotein C-III gene (10)]. Unlabeled competitor oligonucleotides were added in amounts (molar excess) as indicated.

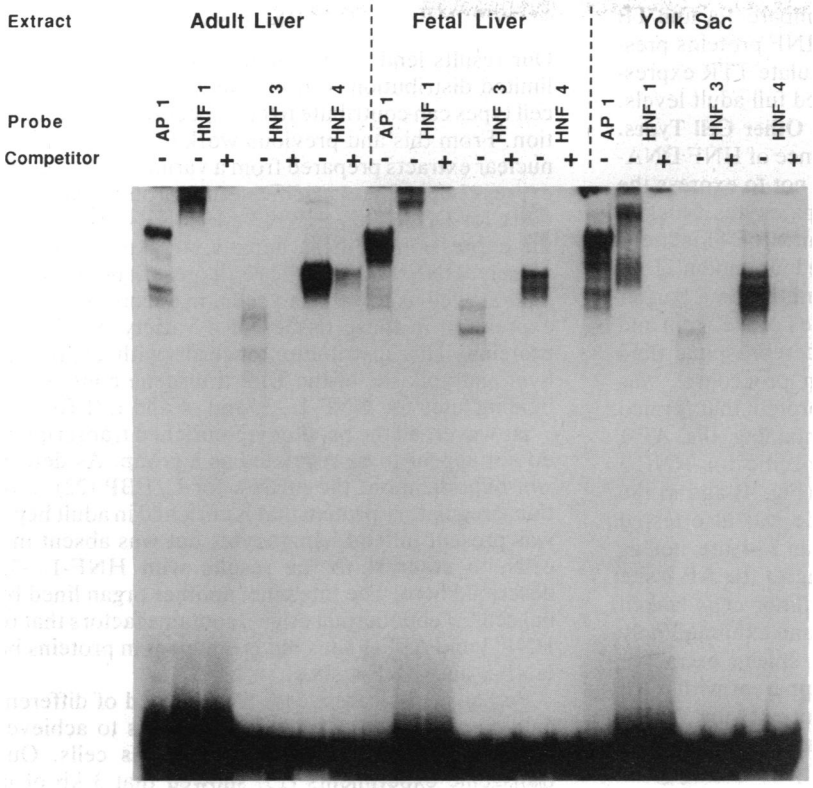

FIG. 2. Presence of HNF binding activity in nuclear extracts prepared from fetal liver and visceral yolk sac correlates with expression. Nuclear extracts of adult liver, fetal liver, or visceral yolk sac (isolated from 17-day embryos) were used in gel retardation assays as in Fig. 1. The labeled probe is given above the lanes [HNF-1, −90 to −61 of the rat β-fibrinogen gene (9)] and unlabeled homologous competitor was added where indicated (+). The AP-1 oligonucleotide (−297 to −272 of the α_1-antitrypsin gene (oligo B in ref. 12) was used as a control to show that active DNA-binding proteins were present in the extracts, since it is a widely distributed factor (11).

assay was used that simultaneously measures transgene and endogenous TTR gene expression (13). With both constructs yolk sac expression was from 30% to 120% that of the endogenous gene, indicating that the minimal protein binding sites necessary for adult liver expression are also sufficient for yolk sac expression, perhaps driven by three of the four liver-specific proteins present in these cells (Fig. 2). The transgene in fetal liver gave clearly detectable but reduced expression (from 10% to 35% of endogenous gene expression). The average expression in fetal liver was higher in mice

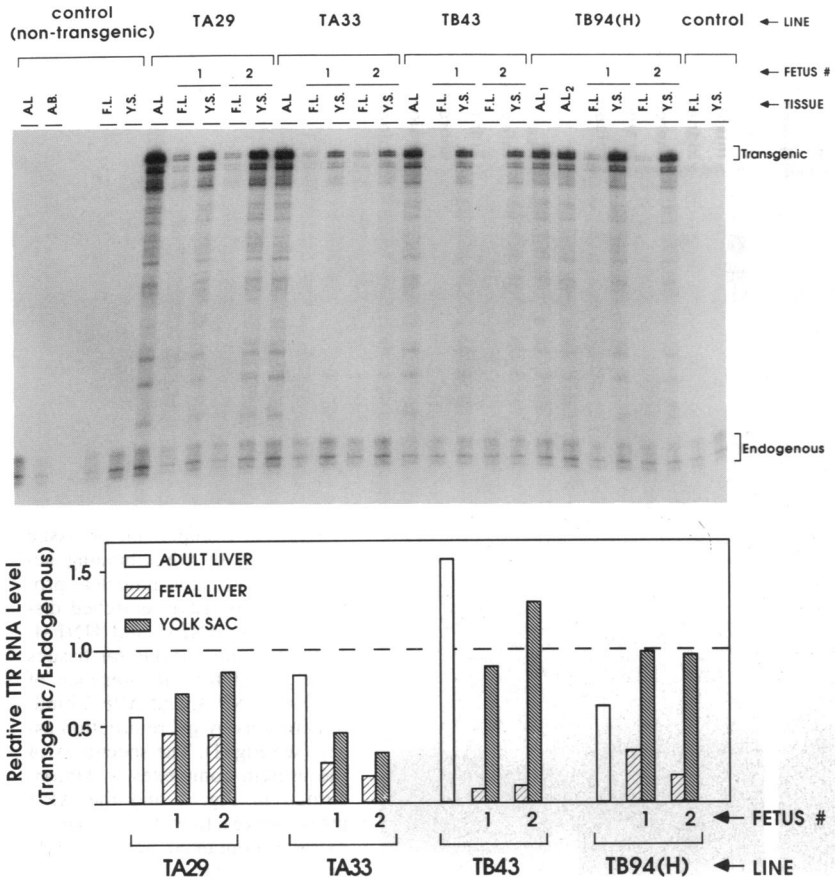

FIG. 3. Transgenic expression in visceral yolk sac and fetal liver directed by TTR regions. Transgenic mice containing TTR regulatory sequences fused to a reporter construct were analyzed by an RNase T2 protection assay for mRNA expression (13) from the transgene and endogenous TTR gene in the adult liver, fetal liver, and yolk sac. The level of transgene expression compared to endogenous gene expression in each tissue is presented. TA mice contained transgene with 3 kb of upstream TTR sequence and TB mice contained only the 100-nucleotide enhancer and 202 nucleotides of upstream sequence from the TTR gene (13).

with the 3-kb TTR constructs (Fig. 3, compare TA and TB animals). These results suggest that the HNF proteins present in fetal liver may also be used to stimulate TTR expression in fetal cells but may not have reached full adult levels.

Distribution of Transcription Factors in Other Cell Types. The next experiments compared the presence of HNF DNA-binding proteins in several tissues known not to express the large group of proteins characteristic of hepatocytes. Nuclear protein extract was also made from a simian virus 40-induced choroid plexus papilloma that produced abundant TTR mRNA (23). The choroid plexus epithelium forms a barrier surrounding vascular tissue in the ventricles of the brain and secretes protein including TTR into the cerebrospinal fluid (4). The adequacy of nuclear extraction procedures was tested by the presence in each extract of protein that formed complexes with the oligonucleotide containing the AP-1 binding site. Labeled oligonucleotides specific for HNF-3 and -4 were used in the first experiment (Fig. 4) and in the second, an HNF-1-specific oligonucleotide was also tested (data not shown). The proteins HNF-3 and -4 are not as widely distributed as is the protein binding to the AP-1 site (Fig. 4). Extracts of the choroid plexus tumor cells lacked proteins that bind the HNF-3 and -4 sites and exhibited only weak affinity for APF-1 activity (as did spleen extracts). HNF-3 and HNF-4 complexes were not apparent with most of the nonhepatic extracts tested. In contrast, the APF-1 probe bound almost equally well with intestine and kidney extracts as with liver extracts, whereas only the kidney gave a weak binding signal with the HNF-4 probe. Finally, we found that HNF-1 activity was also missing from choroid plexus cell extracts but was present in intestinal extracts. The latter result was also recently reported by Baumhueter *et al.* (17).

DISCUSSION

Our results lend strong support to the general thesis that a limited distribution of transcription factors among different cell types can contribute to cell-specific differential transcription. From this and previous work (6, 16, 17) it is clear that nuclear extracts prepared from a variety of tissues that do not express hepatocyte-specific serum proteins lack binding activity for DNA sites (HNF-3 and HNF-4) that are critical to the expression of these hepatocyte-expressed genes. Conversely, HNF-1, -3, and -4 are all present in the adult and fetal liver as well as in yolk sac cells, in accord with the common expression in these tissues of a variety of secreted serum proteins. This distribution accords with expression in the liver and yolk sac of the TTR transgene containing only the binding sites for HNF-1, -3, and -4 and C/EBP.

However, all the hepatocyte-enriched transcription factors do not appear to be regulated as a group. As detected by *in situ* hybridization, the mRNA for C/EBP (22), a transcriptional regulatory protein that is enriched in adult hepatocytes, was present in fetal hepatocytes but was absent in yolk sac cells, in contrast to the results with HNF-1, -3, and -4 described here. The intestine, another organ lined by epithelial cells of endodermal origin, contains factors that recognize HNF-1 and APF-1 sites but is deficient in proteins binding to HNF-3 and HNF-4 sites.

Perhaps the clearest case for the need of different combinations of factors in different cell types to achieve specific expression comes from choroid plexus cells. Our earlier transgenic experiments (13) showed that 3 kb of upstream sequence of the TTR gene allowed nearly normal transcription of transgenes in the choroid plexus whereas the upstream enhancer of 100 nucleotides and 200 nucleotides adjacent to the RNA start site did not. Hepatocytes expressed the TTR sequence in either case. This implies that within this 3 kb of

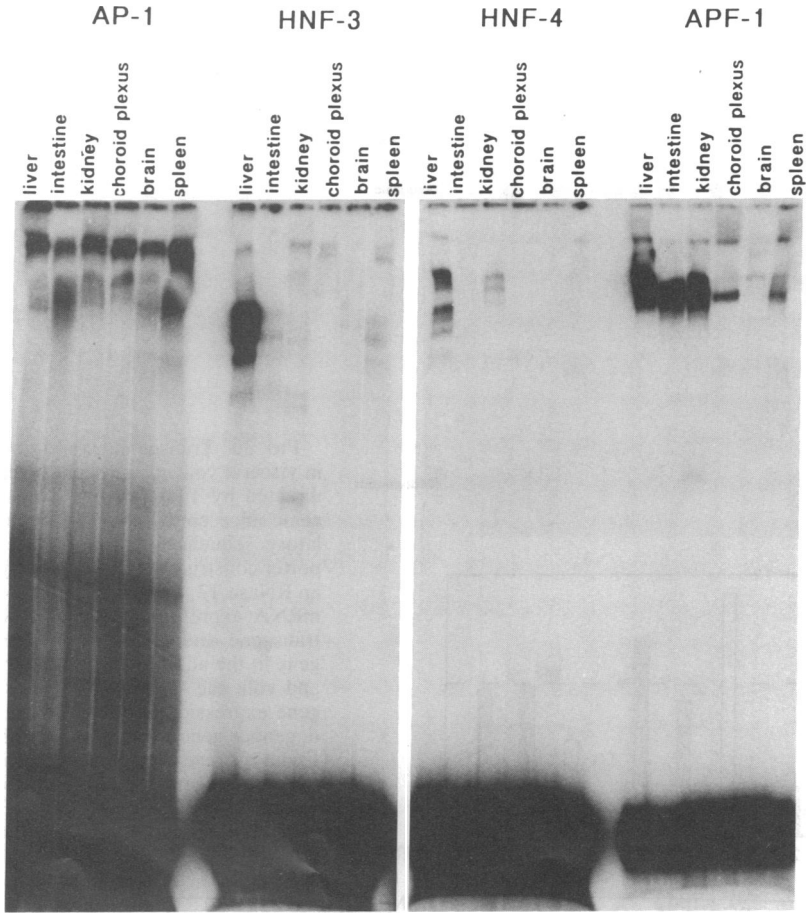

FIG. 4. Comparison of tissue distribution of DNA-binding activity to HNF sites in nuclear protein extracts. Liver-enriched distribution of HNF-3 and HNF-4. Extracts from various rat tissues were tested for the presence of HNF-3, HNF-4, and APF-1 binding proteins by gel retardation assay (see Fig. 1). The specificity of the protein complexes is demonstrated in Fig. 1 and the AP-1 probe serves to indicate that all extracts contained active DNA-binding proteins.

TTR upstream region there must be binding sites for proteins present in choroid plexus nuclei that are not needed to activate TTR in the liver. In support of this hypothesis HNF-1, -3, and -4 were all absent from nuclear extracts of choroid plexus tumor cells. In addition C/EBP was not observed by *in situ* hybridization in the choroid plexus (22). It seems reasonable that the expression of TTR in the choroid plexus must employ different DNA regulatory sequences and factors to stimulate transcription from the identical TTR initiation site that is used in the liver. These results might explain why hepatocyte-expressed genes other than TTR that are regulated by HNF-1, HNF-3, or C/EBP sites are silent in choroid plexus cells.

While these results provide some insight into cell-specific expression, a number of puzzles remain. In most cases the individual binding sites in the various genes that have been studied by transfection analysis in hepatomas appear to act additively. Furthermore, only a few of the sites may contribute disproportionately to the final rate of transcriptional activity (6, 9, 10, 24, 25). If these rules were uniformly obeyed in the adult animal and the expression level were entirely dependent on the proportional representation of factors revealed in cell extracts, then gene control at the transcriptional level would not be as strict as it is. For example, the presence of some HNF-1 in intestine, HNF-4 in kidney, and APF-1 in several tissues is not associated with 5–10% levels of expression of all hepatocyte-expressed mRNAs in these tissues. Other circumstances, probably including negatively acting factors (13, 26, 27), as well as adequate concentrations of positively acting factors at critical moments of development, also may play an important role in the overall scheme of cell-specific regulation. Thus the identification of necessary positive-acting factors is only the first step in explaining cell-specific transcription.

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1. Derman, E., Krauter, K., Walling, L., Weinberg, C., Ray, M. & Darnell, J. E., Jr. (1981) *Cell* **23**, 731–739.
2. Powell, D. J., Friedman, J., Oulette, A. J., Krauter, K. & Darnell, J. E., Jr. (1984) *J. Mol. Biol.* **179**, 21–35.
3. Meehan, R. R., Barlow, D. P., Hill, R. E., Hogan, B. L. M. & Hastie, N. D. (1984) *EMBO J.* **3**, 1881–1885.
4. Schreiber, G. (1987) in *The Plasma Proteins, Structure, Function and Genetic Control*, ed. Putnam, F. W. (Academic, New York), Vol. 5, pp. 293–363.
5. Soprano, D. R., Soprano, K. J. & Goodman, D. S. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 7330–7334.
6. Costa, R. H., Grayson, D. R. & Darnell, J. E., Jr. (1989) *Mol. Cell. Biol.* **9**, 1415–1425.
7. Fried, M. & Crothers, D. M. (1981) *Nucleic Acids Res.* **9**, 6505–6525.
8. Garner, M. & Revzin, A. (1981) *Nucleic Acids Res.* **9**, 3047–3060.
9. Courtois, G., Morgau, J. G., Campbell, L. A., Fourel, G. & Crabtree, G. R. (1987) *Science* **238**, 688–692.
10. Reue, K., Leff, T. & Breslow, J. L. (1988) *J. Biol. Chem.* **263**, 6857–6864.
11. Lee, W., Haslinger, A., Karin, M. & Tjian, R. (1987) *Nature (London)* **325**, 368–372.
12. Grayson, D. R., Costa, R. H., Xanthopoulos, K. G. & Darnell, J. E., Jr. (1988) *Science* **239**, 786–788.
13. Yan, C., Costa, R., Darnell, J. E., Jr., Chen, J. & Van Dyke, (1990) *EMBO J.* **9**, 869–878.
14. Costa, R. H., Lai, E. & Darnell, J. E., Jr. (1986) *Mol. Cell. Biol.* **3**, 1552–1561.
15. Costa, R. H., Grayson, D. R., Xanthopoulos, K. G. & Darnell, J. E., Jr. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3840–3844.
16. Frain, M., Swart, G., Monaci, P., Nicosia, A., Stampfli, S., Frank, R. & Cortese, R. (1989) *Cell* **59**, 145–157.
17. Baumhueter, S., Mendel, D. B., Conley, P. B., Kuo, C. J., Turk, C., Graves, M. K., Edwards, C. A., Courtois, G. & Crabtree, G. R. (1990) *Genes Dev.* **4**, 372–379.
18. Landschultz, W. H., Johnson, P. F., Adashi, E. Y., Graves, B. J. & McKnight, S. L. (1988) *Genes Dev.* **2**, 786–800.
19. Landschultz, W. H., Johnson, P. F. & McKnight, S. L. (1988) *Science* **240**, 1759–1764.
20. Colantuori, V., Pirozzi, A., Blance, C. & Cortese, R. (1987) *EMBO J.* **6**, 631–636.
21. Grayson, D. R., Costa, R. H., Xanthopoulos, K. G. & Darnell, J. E., Jr. (1988) *Mol. Cell. Biol.* **8**, 1055–1066.
22. Kuo, C. F., Xanthopoulos, K. G. & Darnell, J. E., Jr. (1990) *Development* **109**, 473–481.
23. Van Dyke, T. A., Finlay, C., Miller, D., Marks, J., Lozano, G. & Levine, A. J. (1987) *J. Virol.* **61**, 2029–2032.
24. Monaci, P., Nicosia, A. & Cortese, R. (1988) *EMBO J.* **7**, 2075–2087.
25. Cereghini, S., Raymondjean, M., Carranca, A. G., Herbomel, P. & Yaniv, M. (1987) *Cell* **50**, 627–638.
26. Hammer, R. E., Krumlauff, R., Camper, S. A., Brinster, R. L. & Tilghman, S. M. (1987) *Science* **235**, 53–58.
27. Herbst, R. S., Friedman, N., Darnell, J. E., Jr., & Babiss, L. E. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 1553–1557.