p21waf1 mRNA Contains a Conserved Element in Its 3′-Untranslated Region That Is Bound by the Elav-like mRNA-stabilizing Proteins*

Benjamin Joseph, Martin Orlan, and Henry Furneaux†

From the Program in Molecular Pharmacology and Therapeutics, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

The Elav-like proteins are specific mRNA-binding proteins that regulate mRNA stability. The neuronal members of this family (HuD, HuC, and Hel-N1) are required for neuronal differentiation. In this report, using purified HuD protein we have localized a high affinity HuD binding site to a 42-nucleotide region within a U-rich tract in the 3′-untranslated region of p21waf1 mRNA. The binding of HuD to this site is readily displaced by an RNA oligonucleotide encoding the HuD binding site of c-fos. The sequence of this binding site is well conserved in human, mouse, and rat. HuD mRNA. p21waf1 is an inhibitor of cyclin-dependent kinases and proliferating cell nuclear antigen and induces cell cycle arrest at G1/S, a requisite early step in cell differentiation. The identification of an Elav-like protein binding site in the 3′-untranslated region of p21waf1 provides a novel link between the induction of differentiation, mRNA stability, and the termination of the cell cycle.

The Elav-like proteins are tumor antigens that are the human homologues of the Drosophila protein Elav (1). There are four members of the human Elav-like family, namely HuD, HuC, Hel-N1, and HuR (1–5). HuD, HuC, and Hel-N1 are exclusively expressed in post-mitotic neurons and in neuroendocrine tumors. (4, 6, 7). Elav was originally defined in studies of mutants that were defective in neural function (8, 9). In Elav mutant flies, neuroblasts fail to completely differentiate into neurons and result in embryonic lethality. It is thought that HuD, HuC, and Hel-N1 are also involved in neuronal differentiation, since they are expressed on terminations of the neuroblast cell cycle (10–12). Indeed, recent evidence has shown that HuD is necessary for neuronal differentiation.1 Treatment of PC12 cells with nerve growth factor leads to cessation of the cell cycle. In nerve growth factor-treated PC12 cells, it is thought that the cessation of the cell cycle is primarily mediated by the induction of p21waf1 (25, 26). p21waf1 inhibits cyclin-dependent kinases and thereby induces cell cycle arrest at G1/S (27–29). It is known that p21waf1 mRNA has a short half-life and can be regulated at the posttranscriptional level (30–34). Thus p21waf1 may be the connection between the Elav-like proteins and the termination of the neuroblast cell cycle. In this paper we show that HuD binds to a conserved U-rich element within the 3′-UTR2 of p21waf1 mRNA.

MATERIALS AND METHODS

Preparation of Labeled RNA Transcripts—p21-waf1 plasmid DNA was digested with the appropriate restriction enzymes and transcribed with T7 RNA polymerase in the presence of α-[32P]uridine triphosphate (Amersham Pharmacia Biotech), as described previously (35). p21waf1 was linearized with Smal, yielding a full-length transcript of 2121 nucleotides. p21waf1 was linearized with Pst I, yielding a transcript of 383 nucleotides containing the 5′-UTR and part of the open reading frame. p21waf1 was linearized with BsrEI, yielding a transcript of 622 nucleotides containing the 5′-UTR, open reading frame, and 52 nucleotides of the 3′-UTR. p21waf1 was linearized with BsrEI, yielding a transcript of 1116 nucleotides containing the 5′-UTR, open reading frame, and 546 nucleotides of the 3′-UTR. DNA templates for the p21waf1 fragments AC, A, B, C, and BsrEII-end were synthesized by polymerase chain reaction using the following oligonucleotide primers: for subfragment AC, corresponding to 3′-UTR nucleotides 636–1122, the oligonucleotide (T7A5) TAATAGGACTCATATAGGCTTATGCTAGGTTG and (C3) GGTCCACCTGGCCTTCA. For subfragment A, corresponding to 3′-UTR nucleotides 636–789, the oligonucleotides were (T7A5) ACTCTTAGAACCTCTGCTTCA and (A3) TTCTTAAAGAGACCCTC. For subfragment B, corresponding to 3′-UTR nucleotides 767–893, the oligonucleotides were (T7B5) TCTTAAAGAGACCCTC and (A3) TTCTTAAAGAGACCCTC.
RESULTS

HuD Binds to p21waf1 mRNA—The structure and sequence of the full-length p21waf1 mRNA (p21/Smal, 2121 nucleotides) is shown in Fig. 1. In the forthcoming text we will refer to the mRNA as only p21. This will simplify our references to the various subsegments. The transcript encoding this message is too large to assay using conventional gel retardation assays. Thus the RNase T1 selection assay (20) was used to ascertain the RNase T1 selection assay (20) was used to ascertain the segments of the transcript that bound HuD.

Three fragments of 24, 16, and 15 nucleotides were selected from the full-length transcript (p21/Smal, Fig. 1A) by HuD (Fig. 2A, lane 2), whereas no fragments were selected by a GST protein (lane 1), used as a negative control in this experiment. To map these binding sites more precisely, we used this assay to examine two truncated transcripts, p21/Pst and p21/BstEII (Fig. 1A). The p21/BstEII transcript binds fragments of the same size as does the full-length transcript (p21/Smal), although the p21/PstI transcript fails to bind any fragments (Fig. 2A, lanes 2, 5, and 8, respectively). These data suggest that the HuD binding site lies between the PstI and BstEII sites. To confirm this, we used the RNase T1 selection assay to analyze the three adjacent transcripts, p21/BsrBI, p21/BsrBI-BstEII, and p21/BstEII-Smal (Fig. 1A), that were synthesized to span the entire length of the p21 message. Since only the p21/BsrBI-BstEII transcript yielded the selected fragments (Fig. 2B, lanes 4–6), the HuD binding site was localized to a region in the 3′-UTR between the BsrBI and BstEII sites.

Quantitative Determination of the Affinity of HuD for p21waf1 mRNA—To quantitate the relative binding affinities of HuD to p21waf1 mRNA, we employed the method originally used for the R7 coat protein (36). A low amount of labeled RNA (350 pmol) was incubated with HuD protein under conditions of protein excess. The reactions were filtered through nitrocellulose, and the bound radioactivity was measured. Fig. 3A shows that the...
formation of p21/Bsr-BstEII RNA-HuD complex is first detectable at a HuD concentration of 0.15 nM and plateaus at a concentration above 500 nM with about 72% of the input RNA bound. In contrast, complex formation with p21/BsrBI was not detectable even at 1000 nM HuD. A plot of the log of complex/free RNA versus the log of HuD concentration is shown in Fig. 3B. From this plot, the \( K_d \) of the p21/BsrBI-BstEII-HuD complex was determined to be 62.7 nM. Thus the affinity of HuD for p21 mRNA is similar to what we determined for c-fos mRNA (\( K_d = 19 \) nM) (20).

Mapping of the HuD Binding Site—We next examined the sequence of the p21/BsrBI-BstEII transcript (nucleotides 636–1122, Fig. 1B) to delineate the likely location of the 15-, 16-, and 24-nucleotide binding sites. Based on this analysis, we divided p21/BsrBI-BstEII segment into three contiguous transcripts: A, B, and C. Transcript A contains a 16-nucleotide RNase T1 fragment separated from a 24-nucleotide RNase T1 fragment by two nucleotides, whereas B contains two adjacent 15-nucleotide fragments, and C contains only a single 16-nucleotide fragment. Transcripts A, B, C, and p21/BsrBI-BstEII were then tested for HuD binding by gel retardation analysis (Fig. 4A). Purified recombinant HuD was incubated with labeled transcript and assayed for complex formation. HuD binds both p21/BsrBI-BstEII and A with similar affinity (lanes 3–5 and 8–10, respectively) and with a considerably lower affinity to B (lanes 13–15). No binding to C was observed within the range of HuD concentrations tested (lanes 18–20). As expected, no complex formation was observed with transcripts A, B, or C when HuD was replaced by GST (lanes 2, 7, 12, and 17). These semiquantitative observations were confirmed by nitrocellu-

**Fig. 2.** HuD binds to elements in the 3'-UTR of p21 mRNA. A, \( ^{32} \)P-labeled p21/Sma (lanes 1–2), p21/BstEII (lanes 4–5), and p21 Pst (lanes 7–8) transcripts (4 fmol, 20,000 cpm/pmol of UTP) were incubated with 50 nM HuD or GST as indicated at 37 °C for 10 min. After treatment with RNase T1 (5 units/reaction) at 37 °C for 10 min, the reaction mixtures were filtered through nitrocellulose. Bound RNA fragments were extracted and electrophoresed on a 12% acrylamide, 50% urea gel. Lanes 3, 6, and 9 show RNase T1 digestions of the indicated transcripts before nitrocellulose filtration. Lane M, size markers, \( dX174/Hin\) digest. B, same as A, except with \( ^{32} \)P-labeled p21/BsrBI (lanes 1–3), p21/BsrBI-BstEII (lanes 4–6), and p21/BstEII-Sma (lanes 7–9) transcripts (7 fmol, 45,000 cpm/pmol of UTP).

**Fig. 3.** Quantitation of the affinity of HuD for regions of p21 mRNA 3'-UTR. RNA-protein complex formation was assayed by nitrocellulose filtration. \( ^{32} \)P-Labeled transcripts as indicated (14 fmol, 45,000 cpm/pmol of UTP) were incubated with the indicated concentrations of HuD at 37 °C for 10 min. A, a plot of the percentage of RNA bound versus the log of HuD concentration. B, plot of the log of complex/free RNA versus log of HuD concentration. □, p21/BsrBI-BstEII; ◦, p21/BstEII.
lose binding assays by which the $K_d$ values of p21/BsrBil-BstEII, A, B, and C were determined to be 62.7, 85.6, 757, and 10,700 nM, respectively. On the basis of these data, it is likely that transcript A contains the major HuD binding site within the 3' UTR of p21 waf1 mRNA. A minor HuD binding site appears to also be present in B, whereas no site with significant affinity for HuD is observed in C.

To confirm the location of the HuD binding sites within p21waf1 mRNA, transcripts A and B were further analyzed by the RNase T1 selection assay (Fig. 4B). Fragments of 24 and 16 nucleotides in length were selected after incubation of transcript A with HuD, whereas a 15-nucleotide fragment was the predominant species selected after the binding of transcript B. 

Fig. 4. Fine mapping of the HuD site. A, $^{32}$P-labeled p21/BsrBil-BstEII (lanes 1–5), A (lanes 6–10), B (lanes 11–15), and C (lanes 16–20) transcripts (7 fmol, 45,000 cpm/pmol of UTP) were incubated with the indicated concentrations of GST or HuD. After incubation at 37 °C for 10 min, 20% of the reaction mixtures were resolved by gel electrophoresis on a 1% agarose gel. B, $^{32}$P-labeled p21/BsrBil (lanes 1–2), A (lanes 4–5), and B (lanes 7–8) transcripts (7 fmol, 45,000 cpm/pmol of UTP) were incubated with 50 nM HuD or GST as indicated at 37 °C for 10 min. After treatment with RNase T1 (5 units/reaction) at 37 °C for 10 min, the reaction mixtures were filtered through nitrocellulose. Bound RNA fragments were extracted from the filter. RNA (lanes 7–12) was also incubated with the indicated amounts of RNase T1 at 37 °C for 10 min. 10% of the reaction mixtures were electrophoresed on a 12% acrylamide, 50% urea gel.

Fig. 5. The effect of RNase T1 concentration on the selection of HuD binding sites. A, lanes 1–6, $^{32}$P-labeled p21/BsrBil-BstEII (7 fmol, 45,000 cpm/pmol of UTP) was incubated with 100 nM HuD or GST as indicated at 37 °C for 10 min. After treatment with the indicated amounts of RNase T1 (lanes 7–12) at 37 °C for 10 min, the reaction mixtures were filtered through nitrocellulose. Bound RNA fragments were extracted from the filter. RNA (lanes 7–12) was also incubated with the indicated amounts of RNase T1 at 37 °C for 10 min. 10% of the reaction mixtures were electrophoresed on a 12% acrylamide, 50% urea gel. Lane M, size markers, $\phi$X174/HindIII digest. B, same as for A, except with transcript p21/BsrBI, as a negative control.

observed in Fig. 7, were eluted from a preparative gel and analyzed after further digestion with RNase T1, 0.5 units/reaction (lanes 2, 4, and 6, respectively) and with no further RNase T1 digestion (lanes 1, 3, and 5, respectively). Unbound transcript B was treated with RNase T1 (lane 8) or untreated (lane 7) under the same conditions. Lane M, size markers, $\phi$X174/HindIII digest.
The Site in p21 mRNA Is Similar in Structure to Other HuD Sites and Is Well Conserved—We have compared the sequence of the p21 mRNA binding site with those we have found in c-fos, c-myc, tau, GAP-43, and p21 mRNAs (Fig. 6A). The p21 binding site is similar to the others in that it contains U-rich tracts (19, 20, 24). However, unlike other known HuD binding sites, two G residues are present within the site in p21 mRNA, and we obtained two fragments in the selection assay. If the localization of the 24- and 16-nucleotide fragments is correct, we should be able to select a 42-nucleotide fragment from a partial digest of a transcript that lacks a HuD binding site (Fig. 5B). As before, HuD selected fragments from less digested RNA (lanes 3–5, respectively). No new digestion products were observed, thus indicating that the observed fragments are as predicted 24, 16, (transcript A), and 15 nucleotides (transcript B) in length. To ensure that the RNase T1 was used in these experiments was active, the entire transcript B was digested by RNase T1 as a positive control (lane 8). Thus, based on the data obtained by gel retardation, RNase T1 digestion, and nitrocellulose binding assays, we conclude that the major binding site in p21 mRNA is localized to a 42-nucleotide region (nucleotides 657–698) within the 3′-UTR (Fig. 1B, boldface). This site is similar to other HuD binding sites in that it contains U-rich tracts (19, 20, 24).

The importance of the U tract sequences in the p21 site is further underscored by their strict conservation between human, mouse, and rat p21 mRNAs (Fig. 6B).

Next, we tested whether the HuD/p21 mRNA complex would be displaced by the c-fos binding site. The sequence AUUUUAUUUUUUUAUUUUU, termed the c-fos element, has been determined to be the minimal HuD binding site within c-fos mRNA (20). Another oligonucleotide, a mutated c-fos element with the sequence AUACGUAUACGCUACGCUACGCU, fails to bind HuD, served as the negative control in these experiments. As shown in Fig. 7, the addition of unlabeled c-fos element oligonucleotide (lanes 3–5) and 6–8, respectively). After incubation at 37 °C for 10 min, 20% of the reaction mixtures were resolved on a 1% agarose gel.

**DISCUSSION**

The Elav-like proteins have emerged as an important class of proteins that are pleotropic effectors of gene expression in mammalian cells. There are four members of the human family HuD, HuC, Hel-N1, and HuR (1–4). These RNA-binding proteins regulate gene expression via a specific interaction with U-rich elements in mRNA (18, 20–23). These U-rich elements...
are found in a wide variety of mRNAs and usually target them for rapid degradation (17). The current model is that activation of the Elav-like proteins leads to the stabilization of a specific subset of mRNAs. It is likely that this mechanism is responsible for the induction of neuronal differentiation by the neuronal-specific Elav-like proteins. Some of the target mRNAs stabilized by the Elav-like proteins during neuronal differentiation have been identified. Two targets, GAP-43 and tau, are required for the later steps in neuronal differentiation (24). The neuronal Elav-like proteins, however, are expressed before the up-regulation of GAP-43 and tau mRNA. Thus we have looked for new mRNAs that may directly affect the neuroblast cell cycle. In nerve growth factor-treated PC12 cells, there is compelling evidence to suggest that the induction of p21waft, an inhibitor of cyclin-cdk complexes as well as proliferating cell nuclear antigen, plays a critical role in arresting these cells at G1/S, a requisite early step in cell differentiation (25, 26). Thus we examined the interaction between p21waft mRNA and HuD.

In this report, we have localized a high affinity HuD binding site to a conserved 42-nucleotide region within a U-rich tract in the 3'-UTR of p21waft mRNA. Thus, given that the Elav-like proteins regulate other mRNAs with similar sites, it is very likely that the Elav-like proteins will regulate p21waft mRNA. With the detailed knowledge of the p21waft binding site reported here, experiments to directly test this possibility can now be carried out.

Surprisingly, possible changes in the half-life of p21waft mRNA during neurogenesis have not yet been examined. Recent studies, however, in other cell systems have shown that the half-life of p21waft is regulated by diverse signals. In these systems, regulation cannot be due to binding of HuD or any of the other neuronal-specific members of the family. Rather, it is more likely to be mediated by the ubiquitously expressed member of the Elav-like protein family, HuR, which has also been shown to bind to this region of the p21waft message (data not shown). p21waft is up-regulated in p53-deficient promyelocytic HL-60 cells induced to differentiate along the monocytic lineage by phorbol ester or 1α,25-dihydroxyvitamin D3. The connection drawn here between mRNA stability, differentiation and the cell cycle is a novel one. It has been tacitly assumed that transcriptional control is the rate-limiting pathway that defines commitment to differentiation. A distinctive feature of the mechanism postulated here is that the Elav-like proteins are capable of coordinately regulating a subclass of mRNAs. We have recently observed that the Elav-like proteins also bind to p27 mRNA, another negative regulator of the cell cycle. These observations raise the possibility that abrogation of Elav-like protein activity in differentiated cells may coordinately destabilize mRNAs encoding negative regulators of the cell cycle. Indeed an important future goal will be to investigate whether this would lead to reentry into S phase and cell proliferation.

Acknowledgment—We thank Andrew Koff for his very valuable comments on the manuscript.

REFERENCES

1. Szabo, A., Dalmau, J., Manley, G., Rosenfeld, M. R., Wong, E., Henson, J., Posner, J. B., and Furneaux, H. M. (1991) Cell 67, 325–333
2. Sakai, K., Gofuku, M., Kitazawa, Y., Ogasaewara, T., Hirose, G., Yamazaki, M., Koh, C., Yanagisawa, N., and Steinman, L. (1994) Biochem. Biophys. Res. Commun. 199, 1200–1208
3. Ma, W.-J., Cheng, S., Campbell, C., Wright, A., and Furneaux, H. (1996) J. Biol. Chem. 271, 8141–8151
4. King, P. H., Levine, T. D., Fremeau, R. T., and Keene, J. D. (1994) J. Neurosci. 14, 1943–1952
5. Meyer, V. E., Fan, X. C., and Steitz, J. A. (1997) EMBO J. 16, 2130–2139
6. Marusich, H. M., Furneaux, H. M., Henion, P., and Weston, J. A. (1994) J. Neurobiol. 25, 143–155
7. Dalmau, J., Furneaux, H. M., Cordon-Cardo, C., and Posner, J. B. (1992) J. Pathol. 141, 881–886
8. Campos, A. R., Grossman, D., and White, K. (1985) J. Neurogenet. 2, 197–218
9. Homyk, T. J., Ianno, K., and Pak, W. L. (1985) J. Neurogenet. (1985)2, 309–324
10. Wakamatsu, Y., and Weston, J. A. (1997) Development 124, 3449–3456
11. Barami, K., Iversen, K., Furneaux, H., and Goldman, S. A. (1995) J. Neurobiol. 28, 82–101
12. Marusich, H. M., Furneaux, H. M., Henion, P., and Weston, J. A. (1994) J. Neurobiol. 25, 143–155
13. Gorse, M., Wang, X., and Holbrook, N. J. (1998) Mol. Cell. Biol. 18, 1400–1407
14. Greene, L. A., and Tischer, A. S. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 2424–2428
15. Renan, D. J., Query, C. C., and Keene, J. D. (1991) Trends Biochem. Sci. 16, 214–220
16. Shaw, G., and Kamen, R. (1986) Cell 46, 659–667
17. Chen, C. Y. A., and Shyu, A. B. (1995) Trends Biochem. Sci. 20, 465–470
18. Liu, J., Dalmau, J., Szabo, A., Rosenfeld, M., Huber, J., and Furneaux, H. (1995) Neurology 45, 544–550
19. Ma, W. J., Chung, S., and Furneaux, H. M. (1997) Nucleic Acids Res. 25, 3564–3569
20. Chung, S., Jiang, L., Cheng, S., and Furneaux, H. (1996) J. Biol. Chem. 271, 11518–11524
21. Jain, R. G., Andrews, L. G., McGowan, R. M., Pekala, P. H., and Keene, J. D. (1997) Mol. Cell. Biol. 17, 954–962
22. Levine, T. D., Gao, F., King, P. H., Andrews, L. C., and Keene, J. D. (1993) Mol. Cell. Biol. 13, 3494–3504
23. Levy, N., Chung, S., Furneaux, H., and Levy, A. P. (1998) J. Biol. Chem. 273, 6417–6424
24. Chung, S., Ekrich, M., Perrone-Bizzozero, N., Kohn, D. T., and Furneaux, H. (1997) J. Biol. Chem. 272, 6593–6598
25. Yan, G. Z., and Ziff, E. B. (1995) J. Neurosci. 15, 6122–6132
26. Yan, G. Z., and Ziff, E. B. (1995) J. Neurosci. 15, 6200–6212
27. Waga, S., Hannon, G. J., Beach, D., and Stillman, B. (1994) Nature 368, 571–574
28. Harper, J. W., Adams, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. (1993) Cell 75, 805–816
29. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993) Cell 75, 817–825
30. Schwallier, J., Koehler, H. P., Niklaus, G., Loetscher, P., Nagel, S., Fey, M. F., and Tobler, A. (1995) J. Clin. Invest. 95, 973–979
31. Li, X.S., Rishi, A.K., Chao, Z.M., Dawson, M.I., Jong, L., Shroot, B., Reichart, U., Ordonez, J.V., and Koff, A., unpublished
32. Macleod, K. F., Sherry, N., Hannon, G., Beach, D., Tokino, T., Kinzler, K. W., and Vogelstein, B. (1993) Cancer Res. 53, 6055–6062
33. Zeng, Y.-X., and El-Deiry, W. S. (1996) Oncogene 12, 1557–1564
34. Zhang, W., Grasso, L., McClain, C. D., Gambel, A. M., Cha, Y., Travali, S., Deisseroth, A. B., and Mercer, W. E. (1995) Cancer Res. 55, 668–674
35. Macleod, K. F., Sherry, N., Hannon, G., Beach, D., Tokino, T., Kinzler, K. W., Vogelstein, B., and Jacks, T. (1995) Genes Dev. 9, 935–944
36. Furneaux, H. M., Perkins, K. K., Freyer, G. A., Arenas, J., and Hurwitz, J. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4351–4355
37. Carey, J., Cameron, V., de Haseth, P. L., and Uhlenbeck, O. C. (1983) Biochemistry 22, 2601–2610
38. Ely, Z.M., DAWSON, M. I., LI, X.-S., RISHI, A. K., SHEIKH, M. S., HAN, Q. X., ORDONZ, J. V., SHROOT, B., and FONTANA, J. A. (1995) Oncogene 11, 493–504

2 S. Millard, B. Joseph, H. Furneaux, and A. Koff, unpublished observation.