Histone genes display a peak in transcription in early S phase and are ideal models for cell cycle-regulated gene expression. We have previously shown that the transcription factor interferon regulatory factor 2 (IRF-2) can activate histone H4 gene expression. In this report we establish that a mouse histone H4 gene and its human homolog lose stringent cell cycle control in synchronized embryonic fibroblasts in which IRF-2 has been ablated. We also show that there are reduced mRNA levels of this endogenous mouse histone H4 gene in the IRF-2-/− cells. Strikingly, the overall mRNA level and cell cycle regulation of histone H4 transcription are restored when IRF-2 is reintroduced to these cells. IRF-2 is a negative regulator of the interferon response and has oncogenic potential, but little is known of the mechanism of these activities. Our results suggest that IRF-2 is an active player in E2F-independent cell cycle-regulated gene expression at the G1/S phase transition. IRF-2 was previously considered a passive antagonist to the tumor suppressor IRF-1 but can now join other oncogenic factors such as c-Myc and E2F1 that are predicted to mediate their transforming capabilities by actively regulating genes necessary for cell cycle progression.

Interferon regulatory factor 2 (IRF-2)1 and the related factor IRF-1 are negative and positive regulators, respectively, of the interferon response and are implicated in growth control with IRF-2 having oncogenic potential and IRF-1 demonstrating tumor suppressor activity (1–3). One putative mechanism of the oncogenic activity of IRF-2 is that IRF-2 antagonizes the antiproliferative action of IRF-1 by competing for binding sites in the promoters of several growth-suppressing genes (4–6). An alternative hypothesis is that IRF-2, also known as histone nuclear factor M (HiNF-M), plays a positive role in the cell cycle regulation of the human histone H4 gene FO108 (7–10). Unlike many other genes that are regulated at the G1/S transition point such as dihydrofolate reductase and thymidine kinase, FO108 is regulated by an E2F-independent mechanism (11–13). The proximal promoter element of FO108 that interacts with IRF-2 is essential for the peak in transcription of this gene in early S phase and was designated a cell cycle element (CCE) (8). However, other protein factors can also interact with the CCE, namely HiNF-P (H4TF2) and HiNF-D, a CDP/cut, Cdc2, cyclin A, pRb complex (9, 10, 14, 15).

Point mutations in the FO108 promoter that disrupt in vitro binding of any of these factors individually have limited effects on the cell cycle regulation of this gene (8). This result could be due to partial functional redundancy among these factors, the use of alternative binding sites, or differential binding properties of these factors in vivo. Weak interactions of IRF-2 and CDP/cut with alternative binding sites in the FO108 promoter have been observed in electrophoretic mobility shift assays (EMSA). Therefore, to definitively establish the role of IRF-2 in histone H4 transcription regulation, we have performed cell cycle analyses using embryonic fibroblasts from IRF-2 knockout (IRF-2−/−) mice (16).

EXPERIMENTAL PROCEDURES

Stable Cell Lines—NH 3T3 or embryonic fibroblasts derived from IRF knockout mice, maintained as described previously (7), were transfected with a wild type H4 promoter chloromphenicol acetyltransferase (CAT) construct (7) along with pSV-2neo (17) by the calcium phosphate precipitation method (18). 48 h after transfection, cells were maintained in media containing 400 μg/ml Genticin (Life Technologies, Inc.). Resistant colonies were pooled and assayed for CAT enzymatic activity as described (7). To generate the IRF-2 “add-back” cell lines, H4-CAT IRF-2−/− cells were transfected with pcDNA1-IRF-2 (7, 19) along with the hygromycin-resistant plasmid pCEP4 (Invitrogen). 48 h after transfection, cells were maintained in 400 μg/ml Geneticin and 60 units/ml hygromycin B (Calbiochem). Individual resistant colonies that expressed both CAT mRNA and IRF-2 protein were isolated and expanded.

EMSAs—Whole cell extracts from the various cell lines were prepared as described (20). EMSAs were carried out with 10 μg of each extract and 10 fmol of radioactive CCE probe as described previously (7). Where indicated, 1 pmol of unlabeled oligonucleotide competitor DNA (either wild type or mutant CCE as described previously) was included.

Cell Synchronization and DNA Synthesis Assays—Cells maintained as monolayer cultures were synchronized by the double thymidine block method (21). After the final release from block, cells were collected at hourly time points for RNA isolation and DNA synthesis assays by [3H]thymidine uptake (22).

Northern Analysis—Total RNA was isolated using Trizol reagent according to the manufacturer (Life Technologies, Inc.). 10 μg of total RNA from each time point was separated in a agarose/formaldehyde gel as described (18), blotted on Zeta-Probe (Bio-Rad), and probed with a rat histone H4 fragment (22).

RNase Protection and S1 Nuclease Assays—Antisense RNA probes for CAT and cyclophilin (internal control) were prepared from pTricAT and pTriCyclophilin plasmids using a MaxiScript kit according to the

1 This work was supported by National Institutes of Health Grant GM 32010. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

2 F. Aziz, manuscript in preparation.

3 T. J. Last, P. S. Vaughan, J. L. Stein, and G. S. Stein, unpublished observations.
manufacturer (Ambion) except that the cyclophilin probe was made with 200 μM UTP and 20 μCi of [α-32P]UTP. RNase protection assays were performed with the RPA II kit according to the manufacturer (Ambion) using 10 μg of total RNA, 8 × 10^5 cpm of CAT probe, and 3.5 × 10^5 cpm of cyclophilin probe and digesting with 0.5 units of RNase A and 5 units of RNase T1.

For S1 analysis, 52-nucleotide probes were synthesized that were complementary to the junction of the unique 5'-untranslated region and the coding region of mouse histone H4-A and H4-B mRNA (H4-A, 5'-CCTCTGCGCAGCATAAGCTAATGTAACTGAAACCCCCCTGGG-TGAGCCATTG-3'; H4-B, 5'-CGACCACGACATAGTTAATCTTCTTACAAGCCCTTGATAGGAAGCTGGTGAACT-3'). These probes were 5'-end-labeled with [γ-32P]ATP with T4 polynucleotide kinase (New England Biolabs). S1 nuclease assays were performed with the S1 assay kit according to the manufacturer's directions for oligonucleotide probes (Ambion). Briefly, 10 μg of total RNA and 1.2 × 10^5 cpm of probe were hybridized overnight at 16 °C and digested with 100 units of S1 nuclease. Fragments protected from RNase or S1 digestion were separated on an 8% denaturing acrylamide gel (Sequagel, National Diagnostics), respectively. Quantitation of protected bands was performed with a STORM 840 PhosphorImager and ImageQuant software (Molecular Dynamics).

RESULTS AND DISCUSSION

To compare the cell cycle regulation of a histone H4 gene in synchronized IRF-21/1 and IRF-22/2 cells, a construct containing the wild type FO108 promoter sequence fused to CAT (7) was stably transfected into NIH 3T3 or IRF-22/2 embryonic fibroblasts. Integration of the H4-CAT in each cell type was assayed by CAT enzymatic activity (data not shown).

The H4-CAT stably transfected lines were synchronized by the double thymidine block method, which arrests cells at the G1/S phase boundary (21). The synchronization was monitored by DNA synthesis assays and Northern analysis of cellular histone H4 mRNA levels performed at hourly time points after release from block (Fig. 1). Northern analysis revealed an 8–10-fold peak in total cellular histone H4 mRNA levels at the onset of S phase (Fig. 1A), which is consistent with previous studies (8, 23, 24). This peak in total histone mRNA levels is due to both transcriptional and post-transcriptional mechanisms, with the latter involving sequences in the histone 3'-untranslated region (reviewed in Ref. 25). Therefore, to monitor only the transcriptional contribution to the regulation of histone H4, we measured CAT mRNA levels by RNase protection, which in this case is a direct measure of the stably integrated human histone H4 promoter activity.

A single protected fragment of CAT mRNA was detected for each time point as seen at the top of Fig. 1B. In the IRF-21/1 NIH 3T3 cells, the levels of CAT mRNA peaked concomitantly with the peak of DNA synthesis and showed a 2.5–3-fold increase over base-line levels (Fig. 1B). This magnitude of increase in transcriptional activity agrees with many previous studies from several laboratories in which a 2–5-fold increase in transcription of histone genes occurred at the onset of S phase both in vivo and in vitro (8, 23, 24, 26–28). In contrast, cyclophilin mRNA, which was used as an internal control, showed no fluctuations in mRNA levels over the synchronized time course (Fig. 1C). These results indicate that human H4 transcription is cell cycle-regulated in mouse cells in a manner similar to the regulation of this H4 promoter in human cells (8).

We next examined the regulation of histone H4 in synchro-
nized H4-CAT IRF-2−/− cells. Strikingly, cells that lack IRF-2 show no transient increase in the levels of CAT mRNA in a synchronized time course (Fig. 2). This result was reproducibly seen in several experiments (Fig. 2, inset). Therefore, IRF-2 is critical for the cell cycle regulation of this promoter.

Interestingly, the presence of IRF-1, which is able to bind and activate transcription of the H4 promoter in vitro (7), is unable to promote the proper cell cycle regulation of the H4-CAT gene in IRF-2−/− cells (Fig. 2). This supports our previous study in which IRF-1 was unable to compensate for IRF-2-mediated activation of the H4 promoter in vivo (7). These results demonstrate the specificity of IRF-2 for this cell cycle-regulated gene.

We directly assessed whether IRF-2 is a key mediator in H4 gene transcription during the cell cycle by reintroducing IRF-2 into the H4-CAT IRF-2−/− cells. These cells were stably transfected with a human IRF-2 expression vector, and colonies were assayed for the presence of IRF-2 by EMSA. Four positive isolates are shown in Fig. 3 along with the other cell types used in this study. As expected, no IRF-2-specific complexes are seen in extracts from IRF-2−/− cells (Fig. 3, lane 4), while at least two protein DNA complexes are seen in extracts from NIH 3T3, IRF-1−/−, HeLa, and the IRF-2 add-back cell lines (Fig. 3, lanes 1–3 and 5–8). Assignment of the complexes from NIH 3T3, IRF-1−/−, and HeLa as specific for IRF-2 are based on oligonucleotide competition and antibody identification (Refs. 7 and 10 and data not shown). The exact migration of the protein-DNA complexes from the cells in which human IRF-2 was introduced into mouse cells varies slightly from both the human HeLa cells and the mouse NIH 3T3 and IRF-1−/− cells, but these complexes are IRF-2-specific as shown by oligonucleotide competition (Fig. 3, lanes 9–11). Therefore, we have successfully reintroduced IRF-2 protein in the IRF-2−/− cells.

In a cell cycle analysis of one of the IRF-2 add-back cell lines, a transient peak of H4-CAT transcription is again concomitant with the peak in DNA synthesis (Fig. 4). Similar results were obtained with all four of the IRF-2 add-back cell lines analyzed (Fig. 4, inset). Therefore, the reintroduction of IRF-2 is sufficient to restore the cell cycle-regulated transcription of the human histone H4 gene in the IRF-2 knockout cells.
Having established that IRF-2 is necessary for cell cycle-regulated transcription of the introduced H4-CAT gene, one would expect an effect on the endogenous histone H4 genes. To test this prediction, we examined the regulation of mouse histone H4 genes in IRF-2−/− and IRF-2+/+ cells. Eukaryotic cells have multiple H4 genes that display nearly identical coding sequences but divergent promoter regions. It is thought that these multiple genes are required to ensure proper chromatin structure in various cell types or stages of cell development. We selected two mouse H4 genes, one of which is the apparent homolog of FO108 (designated A (29)) and another that is quite divergent (designated B (30)). We established the binding properties of the CCE interacting factors HiNF-M/IRF-2, HiNF-P, and HiNF-D to the two mouse promoter sequences by EMSA (Table I). Mouse H4-A has the same binding properties as the human sequence, while H4-B does not interact with IRF-2 and has only weak interaction with HiNF-D. We therefore anticipated that H4-A would be regulated in a manner analogous to the human H4 gene FO108 and that H4-B would not be regulated by IRF-2.

We determined the effect of IRF-2 on endogenous histone mRNA by comparing the expression levels of H4-A and H4-B in proliferating IRF-2−/−, IRF-2 add-back, or IRF-1−/− control cells by S1 nuclease protection analysis using specific oligonucleotide probes complementary to a unique segment of the 5′-untranslated regions of H4-A and H4-B. Fig. 5 shows that endogenous H4-A mRNA levels were approximately 3-fold lower in IRF-2−/− cells compared with the IRF-1−/− cells, which have normal IRF-2 levels (Fig. 5, compare lanes 1 and 3). Furthermore, the levels of H4-A are restored when IRF-2 is reintroduced to these cells (Fig. 5a, compare lanes 1 and 2). In contrast, the H4-B gene, which does not interact with IRF-2, generates similar mRNA levels in all three cell types (Fig. 5a, lanes 5–7).

We then monitored the cell cycle regulation of endogenous histone H4 genes in various cell types. Comparison of H4-A and H4-B mRNA levels during the cell cycle of synchronized IRF-2−/− cells shows that there is a transient increase in H4-A mRNA during the cell cycle although the amplitude of the peak is reduced and slightly delayed when compared with the regulation of the H4-B message (Fig. 6, A and B). This moderate level of cell cycle regulation of histone H4-A message in IRF-2−/− cells is not unexpected, because the post-transcriptional mechanisms of cell cycle regulation discussed above are pre-sumably operational for this gene (while they are not a factor in the regulation of CAT mRNA, which was assayed in Figs. 1, 2, and 4). In contrast, maximal cell cycle regulation of histone

---

**Protein interactions with mouse histone genes: H4-A is the homolog of human histone H4 FO108**

The proximal promoter region including the TATA box of each gene is shown. Identical nucleotides are written in uppercase letters while differences are shown in lowercase. The approximate boundaries of the histone nuclear factor/FO108 DNA interactions as determined by DNase I protection and methylation interference (9, 10) are indicated above the sequence.

| **Histone Nuclear Factor Binding** | M/IRF-2 | P | D |
|----------------------------------|---------|---|---|
| **Human H4 FO108** | GGGCGCTTTTCCGGTTTCATCGTTGCCTAATCTGCGATACCTCCTTATATCTACAGGGGA | + | + | + |
| **Mouse H4-A** | cGGCGCTTTTCAATCTGCGTCCCTCCTCCTATATAGGCCA | + | + | + |
| **Mouse H4-B** | cGCGGCTTTTCAATCTGCGTCCCTCAATATAGGCCA | - | + | +/- |

* Determined by EMSA with oligonucleotide competition as previously described (9, 10).

---

**Fig. 5. IRF-2 is Required for Histone Gene Cell Cycle Regulation**

A, S1 analysis of mouse histone H4-A and H4-B in proliferating IRF-2−/− cells (lanes 1 and 5), IRF-2 add-back cell line 1A3 (lanes 2 and 6), or control IRF-1−/− cells that have normal IRF-2 levels (lanes 3 and 7). Free H4-A and H4-B probes are shown in lanes 4 and 8, respectively. B, quantitation of results presented in A expressed as the ratio of H4-A/H4-B. The average results from three separate experiments are shown along with the S.D.
H4-A (and H4-B, not shown) is seen in the IRF-2-positive IRF-1/2 cells (Fig. 6C). Furthermore, the maximal regulation of mouse histone H4-A (and H4-B, not shown) is restored when IRF-2 is reintroduced to the IRF-2+/2 cells (Fig. 6D). Therefore, IRF-2 plays a role in the overall expression and cell cycle regulation of a subset of the mouse histone H4 genes.

Along with previous results demonstrating that IRF-2 can activate both a growth control gene (7) and a differentiation-specific gene (gp91phox) (31), the present study further implicates IRF-2 as an active participant in growth control and not only a passive antagonist of the tumor suppressor IRF-1. The mechanism by which IRF-2 contributes to cell cycle regulation is not yet known but may involve the recently described phosphorylation of IRF-2 (32, 33). Although IRF-2 directly interacts with the H4 promoter, it is not yet clear whether the IRF-2 effect on cell cycle regulation is direct. IRF-2 may be involved in the recruiting of other factors to the promoter region of the H4 gene. It is very likely that the cell cycle regulators present in the HiNF-D complex (cyclin A, Cdc2, and pRB), which interact with multiple histone genes (14), are necessary components of histone H4 cell cycle regulation.

In conclusion, the results described in this report clearly establish for the first time that the oncogenic transcription factor IRF-2 is necessary for correct cell cycle regulation of histone H4 gene transcription and that IRF-2 is required for the coupling of histone gene expression with S phase progression in an E2F-independent manner.

Acknowledgments—We thank Danielle Lindenmuth, Jack Green, and Elizabeth Buffone for technical assistance and the members of the Stein laboratory for helpful discussions.

REFERENCES
1. Harada, H., Fujita, T., Miyamoto, M., Kimura, Y., Maruyama, M., Furia, A., Miyata, T., and Taniguchi, T. (1989) Cell 58, 729–739
2. Harada, H., Kitagawa, N., Tanaka, N., Yamamoto, H., Harada, K., Ishihara, M., and Taniguchi, T. (1993) Science 259, 971–974
3. Miyamoto, M., Fujita, T., Kimura, Y., Maruyama, M., Harada, H., Sudo, Y., Miyata, T., and Taniguchi, T. (1988) Cell 54, 903–913
4. Beneath, P., Igneron, M., Peretz, D., Revel, M., and Chebath, J. (1987) Mol. Cell. Biol. 7, 4488–4504
5. Tanaka, H., and Samuel, C. E. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 7995–7999
6. Tan, R. S-P., Taniguchi, T., and Harada, H. (1996) Cancer Res. 56, 2417–2421
IRF-2 Is Required for Histone Gene Cell Cycle Regulation

7. Vaughan, P. S., Aziz, F., van Wijnen, A. J., Wu, S., Harada, H., Taniguchi, T., Soprano, K. J., Stein, J. L., and Stein, G. S. (1995) Nature 377, 362–365
8. Ramsey-Ewing, A., van Wijnen, A. J., Stein, G. S., and Stein, J. L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4475–4479
9. van Wijnen A., Ramsey-Ewing, A., Bortell, R., Owen, T., Lian, J., Stein, J., and Stein, G. (1991) J. Cell. Biochem. 46, 174–189
10. van Wijnen, A. J., van den Ent, P. M. I., Lian, J. B., Stein, J. L., and Stein, G. S. (1992) Mol. Cell. Biol. 12, 3273–3287
11. van Wijnen, A. J., van Gurp, M. F., de Ridder, M., Tufarelli, C., Last, T. J., Birnbaum, M., Vaughan, P. S., Giordano, A., Krek, W., Neufeld, E. J., Stein, J. L., and Stein, G. S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11516–11521
12. Blake, M. C., and Azizkhan, J. C. (1989) Mol. Cell. Biol. 9, 4994–5002
13. Kim, Y. K., and Lee, A. S. (1991) Mol. Cell. Biol. 11, 2296–2302
14. van Wijnen, A. J., Aziz, F., Grana, X., DeLuca, A., Desai, R. K., Jaarsveld, K., Last, T. J., Soprano, K., Giordano, A., Lian, J. B., Stein, J. L., and Stein, G. S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12882–12886
15. Dailey, L., Roseman Roberts, S., and Heintz, N. (1988) Genes Dev. 2, 1760–1712
16. Matsuyama, T., Kimura, T., Kitagawa, M., Pfeffer, K., Kawakami, T., Watanabe, N., Kündig, T., Amakawa, R., Kishihara, K., Wakeman, A., Potter, J., Furlonger, C. L., Narendran, A., Suzuki, H., Ohashi, P. S., Paige, C. J., Taniguchi, T., and Mak, T. W. (1993) Cell 75, 83–97
17. Beck, E., Ludwig, G., Auerswald, E. A., Reiss, B., and Schaller, H. (1982) Gene (Amst.) 19, 327–336
18. Ausubel, F. A., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1997) Current Protocols in Molecular Biology, John Wiley & Sons, Inc., New York
19. Palombella, V. J., and Maniatis, T. (1992) Mol. Cell. Biol. 12, 3325–3336
20. Jiang, S.-W., and Eberhardt, N. L. (1995) Nucleic Acids Res. 23, 3607–3608
21. Stein, G. S., Stein, J. L., Lian, J. B., Last, T. J., Owen, T., and McCabe, L. (1994) in Cell Biology: A Laboratory Handbook (Celis, J., ed) pp. 282–287, Danish Centre for Human Genome Research, Academic Press, Inc., San Diego
22. Grimes, S., Weisz-Carrington, P., Daum, H., III, Smith, J., Green, L., Wright, K., Stein, G., and Stein, J. (1987) Exp. Cell Res. 173, 534–545
23. Plumb, M., Stein, J., and Stein, G. (1983) Nucleic Acids Res. 11, 2391–2410
24. Baumbach, L. L., Stein, G. S., and Stein, J. L. (1987) Biochemistry 26, 6176–6187
25. Osley, M. A. (1991) Annu. Rev. Biochem. 60, 827–861
26. Heintz, N., Sive, H. L., and Roeder, R. G. (1983) Mol. Cell. Biol. 3, 539–550
27. DeLisle, A. J., Graves, R. A., Marzluff, W. F., and Johnson, L. F. (1983) Mol. Cell. Biol. 3, 1929–1929
28. Artishevsky, A., Delegeane, A. M., and Lee, A. S. (1984) Mol. Cell. Biol. 4, 2364–2369
29. Seiler-Tyynis, A., and Paterson, B. M. (1987) Mol. Cell. Biol. 7, 1048–1054
30. Meier, V. S., Bohni, R., and Schumacher, D. (1989) Nucleic Acids Res. 17, 795
31. Lue, W., and Skalnik, D. G. (1996) J. Biol. Chem. 271, 23445–23451
32. Birnbaum, M. J., van Zundert, B., Vaughan, P. S., Whitmarsh, A. J., van Wijnen, A. J., Davis, R. J., Stein, G. S., and Stein, J. L. (1997) J. Cell Biol. 66, 175–183
33. Sharf, R., Meraro, D., Azriel, A., Thornton, A. M., Ozato, K., Petriein, E. F., Larner, A. C., Schaper, F., Hauser, H., and Levi, B.-Z. (1997) J. Biol. Chem. 372, 9785–9792