Interactions with Single-stranded and Double-stranded DNA-binding Factors and Alternative Promoter Conformation upon Transcriptional Activation of the Htf9-a/RanBP1 and Htf9-c Genes*

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The murine Htf9-a/RanBP1 and Htf9-c genes are divergently transcribed from a shared TATA-less promoter. Transcription of both genes is initiated on complementary DNA strands and is controlled by cell cycle-dependent mechanisms. The bidirectional promoter harbors a genomic footprint flanking the major transcription start site of both genes. Transient promoter assays showed that the footprinted element is important for transcription of both genes. Protein-binding experiments and antibody assays indicated that members of the retinoid X receptor family interact with the double-stranded site. In addition, distinct factors interact with single DNA strands of the element. Double-stranded binding factors were highly expressed in liver cells, in which neither gene is transcribed, while single-stranded binding factors were highly expressed in liver cells, in single DNA strands of the element. Double-stranded site. In addition, distinct factors interact with the retinoid X receptor family interact with the double-stranded site. In addition, distinct factors interact with the retinoid X receptor family interact with the double-stranded site. In addition, distinct factors interact with the retinoid X receptor family interact with the double-stranded site. In addition, distinct factors interact with the retinoid X receptor family interact with the double-stranded site.

The murine Htf9 locus was isolated by virtue of its association with a CG-rich genomic sequence (1) and mapped to mouse chromosome 16 (2). The locus contains two transcriptional units, Htf9-a and Htf9-c, that are transcribed with opposite polarity from complementary DNA strands (see map in Fig. 1A). The lower strand gene, called Htf9-a, encodes Ran-binding protein 1 (RanBP1) (3, 4), an interacting partner of the Ran

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1 The abbreviations used are: RanBP1, Ran-binding protein 1; TS-1, transcription start 1; HFE, Htf9 footprinted element; CAT, chloramphenicol acetyltransferase; XRX, retinoid X receptor; RAR, retinoic acid receptor; ssG1, single-stranded G-rich DNA-containing complex 1; ssC, single-stranded C-rich DNA-containing complex; bp, base pair(s).

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fertent forms of the HFE element might have indicated that the Htf9 initiation region was organized either in the double-stranded or in the single-stranded conformation in different cellular conditions. Since both Htf9-associated genes are transcribed in a cell cycle-dependent manner, we sought to determine whether the interaction of factors with HFE varies in relation to proliferation and transcription. Our findings show that the double-stranded nucleoprotein complex is efficiently assembled with extracts from liver cells, in which transcription from the Htf9 locus is extremely low or absent, while single stranded DNA binding activities are abundant in cycling NIH/3T3 fibroblasts, in which both Htf9-associated genes are actively transcribed. In addition, the region surrounding HFE acquires an S1-sensitive conformation in vivo in cycling, but not in growth-arrested, NIH/3T3 cells, where transcriptional repression occurs. Therefore, the Htf9 promoter appears to assume alternative conformations in relation to transcription. These data suggest that factors of the RXX family and the newly identified single-stranded binding proteins participate to transcriptional control of the Htf9-associated genes by interacting with alternative forms of the HFE element in different cellular conditions.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—Murine NIH/3T3 embryo fibroblasts (ATCC CRL 1658) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum under 5% CO2 at 37 °C. Asynchronously cycling cells were collected from 60–70% confluent cultures. Proliferation arrest was induced by culturing the cells in medium containing 0.5% fetal calf serum for at least 48 h. To obtain S phase-enriched cultures, cell cycle reentry was induced after starvation by adding 15% fetal calf serum and harvesting 15 h after stimulation. Growth arrest and cell cycle progression were monitored by flow cytometry as described (9). Cell samples were analyzed in a FACStar Plus cytofluorimeter using the WinMDI software (10,000 events/sample).

**Protein Extract Preparation and Fractionation**—Protein extracts were prepared from both NIH/3T3 fibroblast cultures and from livers from 3–4-week-old black C57 mice after nuclei isolation as described previously (8). All buffers and solutions contained 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 50 μg/ml antipain, leupeptin, aprotinin, and pepstatin A. For the experiments utilizing NIH/3T3 nuclei) were isolated by centrifugation through 0.8 M sucrose cushion overlaid by 0.3 M sucrose solution. Proteins were extracted in 0.4 M KCl buffer and sequentially precipitated; pelleted proteins from the 30% ammonium sulfate precipitation were redissolved in D buffer (20 mM Hepes, pH 7.9, 20 mM KCl, 2 mM MgCl2, 0.2 mM EDTA, 20% glycerol), dialyzed, and again precipitated using 60% ammonium sulfate fractionation as used in the phenol-chloroform, precipitated, resuspended, digested with a phosphocellulose column and subjected to chromatography. Recovered fractions were extensively dialyzed against D buffer, subjected to chromatography through DEAE 52 cellulose, and finally recovered by step elution using KCl.

**Gel Shift Assays**—Oligonucleotides were synthesized at Genenco Service (Department of Genetics and Molecular Biology, University “La Sapienza,” Rome), except for the RXX-binding oligonucleotide (DR-1 configuration) and its mutated version (MDF-1), which were from Santa Cruz Biotechnology. Oligonucleotide sequences are indicated in the text. Gel shift reactions were set up with 20–100 pM of [γ32P]ATP-labeled oligonucleotide, 5–7 μg of NIH/3T3 cell or liver extract, 1 μg of poly(dI-dC)·poly(dI-dC), 25 mM Hepes (pH 7.6), 10% glycerol, 1 mM dithiothreitol, 0.5 mM EDTA, and 50 mM KCl for 15 min on ice. Slightly different conditions were used for ssG binding: 1 μg of poly(di- dC)·poly(dI-dC), 500 ng of nonspecific single-stranded DNA, 20 mM Hepes, pH 7.9, 10% glycerol, 1 mM dithiothreitol, 1 mM EDTA, 0.1 mM EGTA, 60 mM KCl, and 0.1% Nonidet-P 40. In certain experiments, sodium deoxycholate (0.8% final concentration) was added either to the protein mixture before addition of the probe, or to the complete reaction, and incubated for 10 min on ice; incubations were terminated by adding Nonidet-P 40 (1.5% final concentration). Where required reactions were preincubated with competitor DNA for 10 min on ice before adding the probe. Supershift reactions were set up as above, except that 2 μl (2 μg) of anti-RXX or anti-retinoic acid receptors (RAR) antibody were added to the binding mixtures and further incubated for 45 min at room temperature. Anti-RXX and anti-RAR specificities are described below.

**Western Blot Assays**—Nuclear protein extracts from cycling and growth-arrested NIH/3T3 cells, as well as from liver fractions, were electrophoresed through 10% SDS-polyacrylamide gel, electroblotted in 48 mM Tris-HCl, pH 8.3, 39 mM glycine, 0.037% SDS, and 20% methanol on nitrocellulose membranes and analyzed by Western blotting using either anti-RXX or anti-RAR antibodies (1:1000 dilution). The anti-RXX (AN-197, Santa Cruz sc-774) reacts with all murine RXX subtypes (RXRA, RXRB, and RXRG), but not with RAR factors; the anti-RAR (M-454, Santa Cruz sc-773) reacts with all RAR subtypes (α, β, γ, and δ) but not with RXX factors. Membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:20,000 dilution), and bands were revealed following the enhanced chemiluminescence protocol (ECL reagents, Amersham Corp.).

**Plasmid Constructs**—The pEa-A and pEa-C constructs were generated by inserting the Earl-AluI fragment from the Htf9 promoter (X05830 sequence) in both orientations upstream of the chloramphenicol acetyltransferase (CAT) sequence; the pEa-A and pEa-C constructs were generated by inserting the Earl-SmaI fragment from the Htf9 promoter in both orientations upstream of the CAT sequence. HFE-mutated constructs carried an oligonucleotide identical to the Earl-AluI fragment, except that HFE was mutated to 5′-GAATTCCTATCTGTTGTCCTACG-3′ and 5′-GCGGGAGAGAGAGATGATGCT-3′ on complementary strands (C → T and G → A mutations are underlined). The fragment was cloned in both orientations upstream of the CAT sequence, yielding the pEa-ma and pEa-mc constructs respectively, or ligated to the AluI-SmaI fragment from the Htf9 promoter to yield the pEa-mAI and pEa-mC constructs. In an independent set of constructs, the HFE oligonucleotide was ligated in multiple copies downstream of the pA10.CAT2 promoter, which carries two copies of the SV40 21-bp repeat (including Sp1 sites) and a TATA box (16).

**Transfections**—NIH/3T3 cells were routinely diluted 1:6 the day before transfection. Typical experiments were carried out using 5 × 105 cells and a mixture composed of DOTAP liposome reagent (Boehringer Mannheim), 5 μg of CAT reporter DNA, and 1 μg of pCMV-lacZ plasmid. Medium was changed 6 h after lipofection; cultures were harvested 36–48 h later. In all experiments, mock-transfected cultures were harvested and analyzed by flow cytometry as a control to verify that cells were actively proliferating. Promoter strengths were quantified by immunoenzymatic staining of the CAT protein (CAT enzyme-linked immunosorbent assay kit, Boehringer Mannheim) and normalized relative to the amount of synthesized β-galactosidase from the co-transfected plasmid (β-galactosidase enzyme-linked immunosorbent assay, Boehringer Mannheim). pSV0 or pCAT promoterless vectors (Promega) were used as negative controls. Four to eight transfections experiments were carried out for each construct.

**Northern Blots**—NIH/3T3 cultures were growth-arrested or synchronized in S phase as described above. Total RNA was extracted following the guanidine-acid phenol method, electrophoresed, stained with ethidium bromide to visualize the 28 and 18 S ribosomal bands, and transferred to GeneScreen membranes as reported in detail elsewhere (9). Probes used for Northern hybridizations were gel-purified fragments corresponding to the Htf9-c, Htf9-a/RanBP1, and glyceraldehyde-3-phosphate dehydrogenase coding sequences.

**S1 Analysis of Plasmids and Nuclear Chromatin**—Plasmid DNAs were prepared using Qiagen columns, which yield approximately 75% of all DNA molecules in the supercoiled form. Plasmids were subjected to S1 digestion in S1 buffer (3 mM ZnCl2, 30 mM sodium acetate, pH 4.5, 30 mM NaCl, and 0.2 mM EDTA) for 30 min at 37 °C. Nuclei from growth-arrested or S phase NIH/3T3 cells were resuspended in buffer A (homo- 

**S1 Analysis of Plasmids and Nuclear Chromatin**—Plasmid DNAs were prepared using Qiagen columns, which yield approximately 75% of all DNA molecules in the supercoiled form. Plasmids were subjected to S1 digestion in S1 buffer (3 mM ZnCl2, 30 mM sodium acetate, pH 4.5, 30 mM NaCl, and 0.2 mM EDTA) for 30 min at 37 °C. Nuclei from growth-arrested or S phase NIH/3T3 cells were resuspended in buffer A (homogenization buffer without sucrose) and digested with increasing amounts of S1 nuclease (Amersham) in S1 buffer as above, except that 300 mM NaCl was used. Digestions were stopped by adding 10 mM EDTA, 10% SDS, and 0.1 mg/ml proteinase K at 37 °C for 5 h. Genomic DNA was repeatedly extracted with phenol and phenol-chloroform, precipitated, resuspended, digested with EcoRI, electrophoresed, and blotted with conventional methods. Terminal probes for indirect end-labeling experiments were purified from the pL2.2 subclone, which contains the Htf9 bidirectional promoter (X05830 sequence); probes were prepared by double digestion either with EcoRI and HindIII, yielding a 315-bp probe from the 3′ region, or with EcoRI and EcoRV, yielding a 485-bp probe from the 5′ region of the Htf9 insert, eluted from preparative gels, uniformly labeled, hybridized to the filter-bound DNA, and washed using standard procedures.
RESULTS

Mutations within the Footprinted Site Flanking TS-1 Impair Promoter Activity—In a previous characterization of the Htf9 locus (shown in Fig. 1A), we mapped the full-length bidirectional promoter to a 273-bp fragment (14), which was subsequently found to contain both basal and cell cycle control elements in each orientation. Deletion of the elements responsible for cell cycle control of each gene yielded a 74-bp fragment, called EA to design the EarI-AluI restriction ends (see Fig. 1B), which directed basal transcription in both directions (7). Genomic footprinting identified functional sites of protein interaction in the fragment (15). Among those, a footprint (framed in Fig. 1B) exactly starts at the nucleotide adjacent to the major transcription start site of both genes (vertical arrows), coding sequences (filled boxes), untranslated regions (empty boxes), and introns (lines). The start codon in each open reading frame is marked and the orientation of transcription is arrowed. B, sequence of the Htf9 promoter in the RanBP1 orientation. Footprinted sites are underlined; identified footprinting factors are indicated above the sequence. The HFE footprint is framed. The EarI, SmaI, and AluI sites used in promoter reporter construction are indicated. C, promoter regions assayed in transient experiments; dotted lines represent deleted regions. Mutations within HFE are represented by the crossed box. The graph on the right shows the relativity activity of constructs; values were calculated in pg of CAT enzyme/100 μg of protein extract and normalized relative to the amount of β-galactosidase from a cotransfected plasmid. Mean values and S.D. were calculated from four to eight experiments for each construct. pSV0 is the empty vector.

An oligonucleotide corresponding to HFE was saturated with mutations (see “Experimental Procedures”) and assayed in either orientation, both in the context of the 74-bp basal promoter (pEA series), and within a larger promoter fragment (180 bp) harboring activator binding sites (pES series); assayed constructs in transient expression experiments in NIH/3T3 cells are shown in Fig. 1C. In the orientation of the Htf9-C gene, HFE inactivation significantly reduced the basal activity of the pEA-C promoter, whose strength decreased by 50%; a milder effect (25% reduction) was recorded in the 180-bp pES-C construct. In the RanBP1 orientation, HFE mutation lowered activity of the 180-bp pES-A promoter by approximately 50%; it was difficult to determine whether the reduction was significant in the pEA-A basal promoter, which worked per se with very low efficiency. Thus, HFE contributes to promoter activity in both orientations, although the effect of mutations is sensed.

Fig. 1. The Htf9 bidirectional promoter. A, map of the murine Htf9 locus, showing the 5’ ends of the Htf9-a/RanBP1 and Htf9-c genes, the major transcription start site of both genes (vertical arrows), coding sequences (filled boxes), untranslated regions (empty boxes), and introns (lines). The start codon in each open reading frame is marked and the orientation of transcription is arrowed. B, sequence of the Htf9 promoter in the RanBP1 orientation. Footprinted sites are underlined; identified footprinting factors are indicated above the sequence. The HFE footprint is framed. The EarI, SmaI, and AluI sites used in promoter reporter construction are indicated. C, promoter regions assayed in transient experiments; dotted lines represent deleted regions. Mutations within HFE are represented by the crossed box. The graph on the right shows the relativity activity of constructs; values were calculated in pg of CAT enzyme/100 μg of protein extract and normalized relative to the amount of β-galactosidase from a cotransfected plasmid. Mean values and S.D. were calculated from four to eight experiments for each construct. pSV0 is the empty vector.
Fig. 2. The HFE site. A, HFE sequence and mutagenized versions; only the strand corresponding to the RanBP1 orientation is shown. Repeats in the wild-type site (WT) are underlined, base substitutions are indicated by asterisks. B, gel shift assays of wild-type HFE with 5 μg of NIH/3T3 extract (lanes 1, 11, and 14) and increasing amounts of wild-type (lanes 2 and 3) or mutated (lanes 4–10) oligonucleotides (50- and 100-fold excess of competitor were used in each set of reactions), after protein extract preincubation with sodium deoxycholate (NaDOC) (lane 12), and after NaDOC addition to the assembled complex (lane 13).

The HFE sequence (5'-GGGTCAAGGGTGTCAGGG-3') harbors a tandem repeat in several combinations, GGTCAGG, GGTCAGC, or TCAGGG. To assess whether HFE acted as an activator-binding element, the pA10.CAT2 vector was used, which contains a minimal promoter composed of two Sp1-binding sites and a TATA box (16). If HFE functioned as a classical modular promoter element, multimerized copies should activate transcription from pA10.CAT2. No difference was instead detected in the efficiency of chimeric promoter constructs bearing one to four HFE copies upstream or downstream of the TATA box, compared with that of pA10.CAT2 alone (data not shown). Thus, the HFE complex does not identify a strong activator-binding element, yet its integrity in the Htf9 promoter is required for full activity in both orientations in NIH/3T3 cells.

Sequence Requirements for Protein Binding to HFE—As an initial step to characterize the factors conferring the protection flanking TS-1, an oligonucleotide was synthesized from the footprinted HFE window and incubated with NIH/3T3 cell extracts. A discrete nucleoprotein complex was detected by gel-shift assays (Fig. 2B, lanes 1, 11, and 14). Since the HFE location near the transcription start is similar to that of certain initiators, competition assays were designed with oligonucleotides characterized for their ability to initiate transcription in TATA-less promoters, including the adenovirus Inr initiator element and binding sites for the YY1, USF, and Sp1 factors (see Weis and Reinberg (15) and references therein). A canonical TATA box was also assayed, since transcription initiation involves direct or indirect interaction of the TBP factor with several TATA-less promoters (17–19). None of the tested sequences interfered with HFE complex assembly; the complex was also insensitive to the addition of anti-TFIID antibodies and failed to interact with purified recombinant TBP protein (data not shown). Thus, the HFE complex does neither include TBP nor characterized initiator-binding proteins.

The HFE sequence (5'-GGGTCAAGGGTGTCAGGG-3') harbors a tandem repeat in several combinations, GGTCAGG, GGTCAGC, or TCAGGG. To define the sequence requirement for protein binding, mutated HFE versions were used as competitors (shown in Fig. 2A). M0 was mutated throughout HFE and used in transient expression experiments (Fig. 1C); M1 and M2 were, respectively, mutated in the distal (leftward) or proximal (rightward) repeat relative to TS-1; M3 carried mutations in the central region of the oligonucleotide which affected both repeats. The results in Fig. 2B show that M3, although carrying only three base substitutions, like M1 and M2, was unable to compete for protein binding to wild-type HFE. In contrast, both M1 and M2, each of which retained one repeat, partly competed with HFE. These experiments indicate that the GGGTCAAGGGTGTCAGGG sequence represents the optimal binding site; the low efficiency of the competition by M1 and M2 shows that their affinity for HFE-binding factor(s) was not completely abolished yet was significantly reduced compared with the wild-type site. These observations suggest that the HFE complex may be stabilized by interactions among proteins binding to adjacent repeats. Indeed, preincubation of protein extracts with sodium deoxycholate, which disrupts weak protein-protein interactions, prevented the complex assembly (Fig. 2B, lane 12); furthermore, deoxycholate addition to the DNA/protein binding reaction disrupted the assembled complex (Fig. 2B, lane 13). The deoxycholate effect was specific because it did not affect the interaction of Sp1, which binds as a monomer, with its DNA target site (data not shown).

HFE Is Recognized by Members of the RXR Family—The repeated structure and sequence of HFE are similar to those contained in gene promoters regulated by retinoic acid, known as retinoic acid response elements, that are composed of a direct repetition of the PuGGTCA motif and are recognized by members of two large families of transcription factors, RARs and RXRs. Searching transcription factor data bases with the HFE sequence depicted the highest degree of raw homology with the H2RII element in the major histocompatibility complex class I gene promoter (Fig. 3A), a target of the RXRβ subtype of retinoid X receptors (20). Unlabeled H2RII interfered with the HFE complex assembly (Fig. 3A, lanes 6–9) almost as effectively as the homologous competitor (Fig. 3, A, lanes 2–5, and C). Reciprocal experiments using labeled H2RII as the probe showed that homologous competitions were highly effective (Fig. 3A, lanes 10–14), while competition by unlabeled HFE required a 50-fold excess to achieve 50% inhibition and plateaued thereafter (Fig. 3A, lanes 15–18, and B). The finding that H2RII was an effective competitor of HFE, while HFE only partially competed for factors binding to the H2RII probe, suggests that H2RII and HFE are bound by both common and specific partners. We next used a canonical RXR-binding oligonucleotide in which PuGGTCA repeats are separated by one-nucleotide spacer (DR-1 configuration), as in HFE; extensive characterization of DR-1 had shown it to be the preferred target sequence of RXR factors (21). Heterologous competitions for factors between DR-1 and HFE were now complete (Fig. 3D); thus, the binding properties of HFE are indistinguishable from those of the DR-1 oligonucleotide. Together, these results implicate RXR factors in binding to HFE.

Distinct RXR-containing Complexes Are Assembled with Extracts from Liver and NIH/3T3 Fibroblasts—Since in previous work the strongest HFE in vitro footprints were detected using
mouse liver extracts (8), the study of HFE-binding factors was further pursued using such extracts. We found that the HFE complex with liver nuclear extracts had higher abundance, and lower electrophoretic mobility, than seen using NIH/3T3 extracts (Fig. 4, compare lanes 1 and 7). Addition of an anti-RXR antibody recognizing all three RXR subtypes (α, β, and γ) to the HFE reaction with liver extracts supershifted the complex in a manner comparable to that seen with DR-1 (Fig. 4, lanes 2 and 5). An antibody recognizing all members (α1, α2, β1, β2, γ1, and γ2) of the RAR family did not affect the HFE nor the DR-1 complex (Fig. 4, lanes 3 and 6). When immunoassays were carried out using the same amount of NIH/3T3 nuclear extract, no clear supershift was detected with either HFE or DR-1 probes; however, the amount of assembled complex with both probes decreased with the addition of both anti-RXR (Fig. 4, lanes 8, 9, 13, and 14) and anti-RAR (Fig. 4, lanes 10, 11, 15, and 16) antibodies, suggesting that component(s) of both the RXX and RAR families interacted with HFE and DR-1 in NIH/3T3 cells. Thus, the differences in abundance, mobility, and antibody reactivity indicate that specific complexes are formed by both probes depending on the extract source.

The results in Fig. 4 suggest that the RXR supershift depicted with liver extracts reflected the higher abundance of these factors in liver, compared with NIH/3T3, nuclei. To further investigate that possibility, liver extracts were fractionated through sequential phosphocellulose and DEAE 52 chromatography (Fig. 5A). All RXR subtypes (α, β, and γ) recognized by the anti-RXR antibody were eluted with, and were abundantly expressed in, fractions that were positive for HFE binding (compare Fig. 5, B and C, upper panel). In NIH/3T3 extracts, only one subtype, whose electrophoretic mobility was compatible with that expected for RXRβ (22), was detected; the relative abundance of the reacted protein was significantly lower than that of the corresponding liver protein, and in cycling NIH/3T3 cells was less abundant than in growth-arrested cultures (Fig. 5C, upper panel, lanes 8 and 9). In NIH/3T3 extracts we also detected RAR factor(s) that had not been depicted in liver cells (Fig. 5C, lower panel). In summary, HFE is bound by RXR factors which associate with different partners and assemble specific complexes with different extract types. Abundant complexes are formed with liver factors, among which RXRs are highly expressed. RXR-containing complexes have lower abundance in NIH/3T3 fibroblasts and include members of the RAR family. In retrospect, the cell type-specific reactivity in supershift assays reflects the different distribution and relative abundance of retinoid receptors depicted in Western blot assays.

Distinct Proteins Bind to Each Strand of HFE in NIH/3T3 Fibroblasts—Since the region surrounding the transcription start of both Htf9 genes can be expected to open up during transcription and expose single-stranded DNA templates to transcriptional complexes, we wondered whether single DNA strands interacted with proteins. The G-rich and C-rich strands of the HFE oligonucleotide were separately incubated with NIH/3T3 extracts and their ability to form nucleoprotein complexes was assessed in gel-shift assays. All forms of the site (i.e. G-rich, C-rich, and double-stranded) assembled discrete complexes (Fig. 6A). The complex formed with the C-rich strand was designated sC (single-stranded C-rich DNA-containing complex). The G-rich strand formed two predominant complexes, ssG1 and ssG2 (single-stranded G-rich DNA-containing complexes 1 and 2) and two fainter complexes of lower mobility. The specificity of the complexes was assessed in competition experiments. The G-rich strand was firstly examined: the assays in Fig. 6B (lanes 1–10) show that none of the competitor DNAs inhibited the faint complexes, which therefore reflect
nonspecific associations. Both ssG1 and ssG2 were instead inhibited by preincubation with homologous (lanes 3 and 4), but not with double-stranded (lanes 8–10), DNA. The complementary strand partially competed for protein(s) (lanes 5–7); however, the excess of C-rich DNA required to inhibit ssG1 and ssG2 formation was at least 5-fold higher than that used in the homologous competition (compare lane 3 to 5, and 4 to 6). Competition experiments were then set up between the C-rich strand and homologous, reverse complementary or double-stranded DNA; ssC assembly was inhibited by preincubation with homologous DNA (lanes 13 and 14), while remaining unaffected in the presence of double-stranded (lanes 18–20) DNA.

Fig. 4. Double-stranded HFE forms distinct complexes in different cell types. The HFE (lane 1) and DR-1 (lane 4) complexes formed with liver extracts are supershifted (arrow) by anti-RXR (lanes 2 and 5) but not anti-RAR (lanes 3 and 6) antibodies; neither HFE (lane 7) nor DR-1 (lane 12) complexes formed with NIH/3T3 extracts are visibly supershifted, yet decrease, with increasing amounts of either anti-RXR (lanes 9, 13, and 14) or anti-RAR (lanes 10, 11, 15, and 16) antibodies. Reactions were set up using 10^6 cpm of labeled probes and 6 μg of nuclear extract from either source.

Fig. 5. Cofractionation of the HFE-binding activity and RXR factors from liver extracts. A, schematic of the procedure used for fractionation of the HFE-binding activity from liver extracts. AS, ammonium sulfate. B, gel-shift assays of HFE with eluted fractions (indicated above each lane). 4 μg of protein from nuclear extract (NE) and phosphocellulose fractions (PC), and 2 μg of DEAE 52 (DE) fractions were used. The addition of homologous competitor DNA is indicated by +. C, Western blot assays of protein from liver fractions and from growth-arrested or cycling NIH/3T3 cells with anti-RXR (top panel) and anti-RAR (lower panel) antibodies.
growth-arrested, compared with cycling, NIH/3T3 cells (Fig. 8, lanes 2 and 3), and highest in liver (Fig. 8, lane 1). G-rich DNA-binding factors showed the reverse pattern, being either more abundant or more active, in cycling compared with quiescent NIH/3T3 cells, and undetectable in liver (Fig. 8, lanes 7–9). The ssC complex was comparatively more widespread, although somewhat less abundant in live cell extracts. Together, the results suggest that different cell types are equipped with distinct sets of factors capable of binding either double-stranded HFE or single DNA strands. Single-stranded complexes most efficiently form with protein extracts from proliferating, i.e. actively transcribing, NIH/3T3 fibroblasts, whereas factors interacting with double-stranded HFE were most abundantly expressed in liver.

Plasmids Containing the HFE Sequence Are Sensitive to S1—Since single-stranded DNA-binding activities were depicted in cell cultures in which the Htf9 locus is transcriptionally active (9), it was important to assess whether single-stranded structures actually formed in the Htf9 promoter.
Computer analysis revealed that HFE fell within one potential stem-and-loop structure; theoretical estimates of the free energy associated with the formation of the single-stranded loop give $\Delta G = -21.7$ kcal/mole. We wondered whether these theoretical sequence features gave rise to the formation of unusual DNA structures in the Htf9 initiation region. Supercoiled plasmids carrying either the full-length (pTS-A, 273 bp), or the minimal (pEA-A, 74 bp), Htf9 promoter were digested with S1 nuclease and subsequently restricted with PstI to separate the Htf9 sequences from the plasmid replication origin (Fig. 9A), which might contain unwound DNA. Results in Fig. 9B show that both the pTS-A and pEA-A constructs contained S1-sensitive sequences that were not present in the vector. Hybridization with labeled HFE oligonucleotide confirmed that the region containing HFE was sensitive to S1 cleavage (Fig. 9C).

To directly ascertain whether HFE in particular represented a DNA target for S1 cleavage, pA10-derived clones carrying one or three HFE copies (Fig. 9D) were subjected to digestion using increasing S1 nuclease doses. While the pA10 vector was inefficiently cleaved by S1 (Fig. 9E, lanes 1–4), plasmids containing one or more HFE copies were sensitive to S1 in a dose-dependent manner. Microdensitometry quantification of the ratio of supercoiled to linear DNA revealed that the extent of cleavage by S1 increased with the number of HFE copies. Thus, the HFE sequence confers S1 sensitivity to supercoiled plasmids.

The Genomic Region Surrounding HFE Is Sensitive to S1 during Transcription in Vivo—We finally assessed whether S1-sensitive structures identified in HFE-containing clones were maintained in the higher order organization of genomic DNA. NIH/3T3 cell cultures were either brought to proliferation arrest by serum withdrawal for 48 h, or stimulated to reenter the cycle by adding fresh serum. Flow cytometric analysis confirmed that cells collected prior to serum stimulation were arrested in the G0/G1 state, while cells collected 15 h after serum refeeding were traversing S phase (Fig. 10A). Neither Htf9-associated gene was transcribed in growth-arrested cells, while transcription was active in S phase cells (Fig. 10B), confirming our previous data on cell cycle control of the Htf9 promoter (7). Nuclei were isolated from both growth-arrested and S phase NIH/3T3 cultures to assess the Htf9 promoter sensitivity to S1 in transcribing and nontranscribing cells. Nuclei were digested with increasing amounts of S1 nuclease; DNA was extracted, restricted with EcoRI and hybridized with

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**FIG. 8.** Distribution of factors binding different HFE forms. Double-stranded HFE (lanes 1–3), C-rich (lanes 4–6), and G-rich (lanes 7–9) oligonucleotides were incubated with protein extracts (4 µg) from liver (lanes 1, 4, and 7), growth-arrested (lanes 2, 5, and 8) and cycling (lanes 3, 6, and 9) NIH/3T3 cells.

**FIG. 9.** S1 sensitivity in HFE-containing plasmids. A, maps of the pSV0-CAT vector and of Htf9-derived promoter constructs, respectively, carrying the basal (pEA-CAT, 74 bp) and the full-length (pTSA-CAT, 273 bp) promoter region. The pBR322 origin of replication (ORI) is marked. P, PstI; H, HindIII. In the series of constructs, the promoter insert (cloned in the HindIII site) is oriented in the direction of RanBP1 transcription relative to the CAT gene. B, ethidium-bromide staining of constructs subjected to digestion with S1 nuclease (lanes 2, 4, and 6) or to mock digestion (indicated by a dash (-) in lanes 1, 3, and 5), and subsequently restricted with PstI to separate the replication origin from the Htf9 promoter region. Lane 7, λ DNA digested with EcoRI and HindIII. C, autoradiograph of the blot from the gel in A after hybridization with labeled HFE oligonucleotide. D, chimeric promoter constructs carrying no (pA10), one (pA10G), or three (pA10G9) HFE copies upstream of the pA10 minimal promoter. The arrow marks the promoter insertion site. E, ethidium bromide-stained gel showing 1 µg of DNA from each construct after digestion with 0, 0.5, 1, and 2.5 units of S1.
a Htf9-derived fragment that was flush with one EcoRI restriction end (map in Fig. 10C). These experiments depicted a restricted region (approximately 100 bp) that was sensitive to S1 cleavage in S phase NIH/3T3 cells (Fig. 10C, lanes 5–7), but not in NIH/3T3 cultures brought to quiescence (Fig. 10C, lanes 1–3). Indirect end-labeling revealed that the S1-sensitive region in S phase cells encompassed the 74-bp region sufficient for basal transcription as functionally defined in promoter assays (see Fig. 1).

DISCUSSION

The HFE Near TS-1 Is Required for Promoter Activity—A 74-bp promoter region carries sufficient information to direct transcription of both the RanBP1 and Htf9-c genes. We have recently begun to characterize the HFE element, originally identified by genomic footprinting, which flanks the TS-1 start site used for initiation of both genes. Promoter sites showing interactions with factors in vivo often identify regulatory elements. HFE is indeed a functional element in both orientations, as mutational inactivation reduces promoter strength in each direction. However, HFE inactivation is sensed differently depending on the orientation and arrangement of neighboring regulatory sequences in the promoter context; HFE inactivation does mildly impair the pES-C promoter, while severely affecting activity of both the pES-A and pEA-C constructs. The effectiveness of regulatory sequences depends, at least in part, on their position in the promoter context. It is possible that regulatory elements included between the pEA-C and pES-C promoter boundaries participate in the assembly of transcriptional complexes and relieve in part the effect of HFE inactivation. Thus, the functional promoter mapping results suggest that the HFE site is involved in mediating interactions among various regulatory elements in each promoter orientation.

RXR Factors Bind the Double-stranded HFE—In the search for factor(s) protecting HFE, we have identified distinct activities which bind different HFE forms. The double-stranded site is recognized by complexes containing retinoid X receptors. These complexes showed cell type-specific differences in both their relative abundance and interacting partners. NIH/3T3 extracts yielded a complex that was similarly reacted by both anti-RXR and anti-RAR antibodies, while liver extracts formed a complex that was specifically reacted by anti-RXR but not anti-RAR antibodies. Thus, RXRs interacting with HFE associate with specific partners in different cells. These findings were paralleled by the distribution of retinoid receptors: in liver nuclei, all three RXR subtypes were abundant and were recovered in chromatographic fractions positive for HFE binding. In NIH/3T3 cells, RAR factors were also depicted, while only one RXR subtype was above the level of detection in Western experiments.

Sites in a DR-1 configuration, such as HFE, can be bound by RXR homodimers or heterodimers involving different partners (reviewed in Refs. 23–25). The DR-1 oligonucleotide used here for control was previously characterized as a preferred target site of RXR homodimers (21). An identical sequence to HFE was also included in an oligonucleotide, designed R7, characterized as a high affinity site for RXRα homodimer binding (26). RXRs are versatile factors that can act as transcriptional activators, be transcriptionally silent although remaining engaged in the interaction with target DNA sites, or contribute to repression in differentiated tissues, as shown for several proliferation-associated genes (27–29), in a ligand-dependent manner (see Minucci and Ozato (30) and references therein).
Immune reactions of both the HFE complexes and of fractions enriched in HFE-binding activity indicate that RXR family members interact with the HFE site in liver. Since transcription from the Rtf9 locus is low or absent in liver cells, unless regeneration is induced by surgical hepatectomy (9), while the binding of RXR complexes is highest compared with other cell types, the interaction of RXR factors with HFE appears to correlate with transcriptional inactivity in liver nuclei.

Western immunoblotting and supershift experiments with NIH/3T3 fibroblast extracts suggest that the RXR subtype(s) in these cells rather heterodimerize with RAR factors while binding HFE. HFE-binding complexes in NIH/3T3 cells have lower abundance than in liver cells, and in cycling NIH/3T3 cells are less abundant than in growth-arrested cultures which cease transcription of the Rtf9-associated genes. We previously noticed that undifferentiated embryo stem cells were the only cell type in which HFE was not footprinted in vivo; embryo stem cell extracts also lacked double-stranded HFE binding activity (15). Since both Rtf9 genes are transcribed in both embryo stem and F9 undifferentiated cells (31), it appears in retrospect that RXR binding is dispensable for expression of the Rtf9 genes during embryonal proliferation. Thus, the binding of RXR-containing complexes to HFE is up-regulated during differentiation and is inversely related to HFE promoter activity.

Specific Factors Bind Each DNA Strand of the HFE—One unexpected HFE feature is that specific activities present in NIH/3T3 cells bind to each DNA strand. That finding prompted us to investigate whether single-stranded structures actually formed in the Rtf9 promoter. Theoretical energy estimates support the possibility that the Rtf9 promoter forms a stem-and-loop structure, and the identification of S1-sensitive sites in HFE-containing plasmids suggests that the formation of single-stranded structures represent an inherent feature of the Rtf9 initiation region. Many CG-rich, TATA-less promoters potentially form single-stranded loops in the region surrounding the transcription start sites (32). Such looping structures have been suggested to serve as structural landmarks and facilitate the recognition of start sites by basal factors in complex genomes, where TATA box recognition by linear sequence scanning would be inefficient compared with smaller genomes (32).

In the Rtf9 bidirectional promoter each DNA strand must serve as a transcriptional template. In vivo sensitivity to S1 was detected in NIH/3T3 cells in which the Rtf9 promoter was active, but not in conditions in which transcription was repressed. The link between proliferation, promoter activity, S1 sensitivity, and expression or activity of the single-stranded binding proteins, suggest that single-stranded proteins identified in this work exert a positive role in transcription. A growing number of single-stranded DNA-binding proteins have now been found to interact with regulatory elements and influence transcription by altering the DNA topology or conformation (33–37). Extensive studies of the c-myc promoter (37–40) are suggestive of a model in which the cellular conditions control the interaction of double-stranded or single-stranded DNA-binding factors to specific promoter elements; the binding of proteins to single DNA strands is thought to induce structural transitions that are sensed as transcriptional signals and mediate c-myc response to cell cycle regulators, growth factors, and other inducing stimuli. On a similar line, two studies of hormone-inducible promoters have shown that the binding of double-stranded and single-stranded binding activities is hormonally regulated and independently mediate control of basal transcription and hormone induction (41, 42). From these studies it appears that the transcriptional response of one same element to inducing or repressing stimuli can be mediated by factors influencing the promoter structural organization and either allow, or prevent, productive interactions among neighboring regulatory factors in different cellular conditions.

It is increasingly clear that RXR complexes, particularly with RARs, can recruit corepressors that cooperate in maintaining a transcriptionally inactive structural organization (43). Ligand addition can destabilize the interaction with corepressors, thereby triggering chromatin remodeling (reviewed in Ref. 44). Various possibilities might be envisaged concerning HFE. RXR complexes might normally occupy HFE and set the TS-1 region in the double-stranded form. In the presence of cell cycle-related stimuli, RXRs could be displaced from the DNA, either directly by the single-stranded binding proteins, or by general factors with helicase activity (12). The single-stranded binding proteins may then facilitate the assembly of initiation complexes on each strand, and/or contribute to looping structures in which activator-binding sites productively interact with the transcription start site. Alternatively, the inherent features of the Rtf9 initiation region might favor the single-stranded conformation, which may represent a natural target for single-stranded binding proteins setting the promoter in a transcriptionally competent state. In cells in which neither Rtf9-associated gene is to be expressed, RXR complexes might catalyze the formation of a double-stranded structure. RXR complexes might not necessarily determine repression in all cells and may exert a negative or a positive function depending on their interactions with positive or negative co-factors (45), the presence of ligands, and the occupancy of adjacent promoter sites by transcriptional repressors or activators.

We have previously shown that cell cycle-dependent transcription of both Rtf9-associated genes is mediated by target elements for both the E2F and Sp1 families of activators, while being repressed by the pRb retinoblastoma protein and its relative p107 (7, 9). The present data show that transcription is also associated with S1 sensitivity and with the interaction of specific proteins with single DNA strands near TS-1, while absence of transcription is associated with loss of S1 sensitivity and increased binding of RXR complexes to the double-stranded HFE. The pRb protein interferes with single-stranded DNA-binding by the Puro protein (46). Thus, the Rtf9 promoter may not only be controlled by the antagonism between retinoblastoma-related factors and E2F activators, but may also involve regulated interactions between single-stranded binding proteins and each DNA strand in the region of initiation, or assumption of the double-stranded conformation associated with the binding of retinoid receptors.

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