Chromosomal localization of nucleic acid-binding proteins by affinity mapping: assignment of the IRE-binding protein gene to human chromosome 9

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ABSTRACT

Three human mRNAs are regulated post-transcriptionally by iron via iron-responsive elements (IREs) contained in each mRNA. A cytoplasmic protein (IRE-BP) binds to these cis-acting elements and mediates the translational regulation of ferritin H- and L-chain mRNA and the iron-dependent stability of transferrin receptor (TfR) mRNA. We have taken advantage of the different mobilities of the human and rodent IRE/IRE-BP complexes on non-denaturing polyacrylamide gels to determine the chromosomal localization of the gene encoding the IRE-BP. Utilizing a panel of 34 different human/rodent hybrid cell lines we have assigned the IRE-BP gene to human chromosome 9. This new technique based on nucleic acid/protein interaction may allow determination of the chromosomal localization of other RNA- or DNA-binding proteins.

INTRODUCTION

Iron-responsive elements (IREs) were originally identified as necessary and sufficient translational regulatory sequences that occur in the 5' untranslated regions (UTRs) of ferritin mRNAs (1−3). Subsequently, they have been recognized in the 3' UTRs of transferrin receptor (TfR) mRNAs and were implicated in the iron-dependent regulation of TfR mRNA stability (4). The cytoplasmic IRE-binding protein (IRE-BP) has been shown to interact with IREs contained within both regulatory regions (5−7). The iron status of the cell determines the ability of the IRE-BP to bind to an IRE through reversible oxidation-reduction of sulfhydryl groups that are critical for the high affinity RNA/protein interaction (8). Thus, the IRE-BP plays a central role in cellular iron homeostasis by regulating ferritin mRNA translation and TfR mRNA stability (9).

Idiopathic hemochromatosis is a potentially fatal hereditary disease which is characterized by a defect in the regulated absorption of iron. The abnormally high duodenal uptake of iron from the diet results in systemic iron overload and consequent damage to multiple organs (10). The genetic defect that underlies this autosomally recessive disorder remains obscure, but linkage analysis has revealed that the affected gene is located on the short arm of human chromosome 6 (6p21) close to the HLA-A locus (11). The important role of the IRE-BP in the regulation of cellular iron metabolism led us to examine its chromosomal localization in view of its potential involvement in the pathogenesis of idiopathic hereditary hemochromatosis.

Traditional approaches for the determination of the chromosomal localization of human genes are based on in situ hybridization of cloned DNA fragments (cDNA or genomic DNA) to metaphase spreads of human chromosomes or the identification of the human gene or gene product in somatic human/rodent cell hybrids. The latter method requires
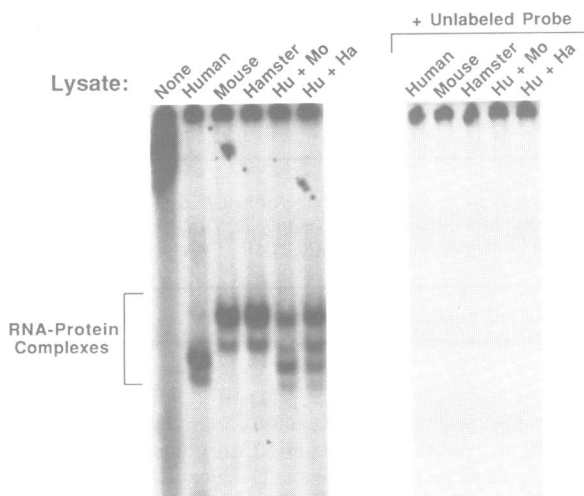
Figure 1. RNA-protein complexes involving human and rodent IRE-BP can be distinguished. Detergent extracts were prepared from RD4 (Human), RAG (Mouse), and E36 (Hamster) cells. The lanes labeled Hu + Mo and Hu + Ha represent samples where extracts from RD4 cells had been mixed with extracts from RAG or E36 cells, respectively, before [^{32}P]-labeled TfR probe was added. In the right panel, a >1000-fold excess of unlabeled TfR probe was added prior to the addition of labeled probe. Autoradiographs of gel retardation assays are shown.

the availability of a cloned DNA probe (12), specific antibodies that distinguish between the human and the rodent gene product of interest (13), or a functional assay with similar capabilities. Since neither the cDNA encoding the IRE-BP nor antibodies against the IRE-BP are available, we utilized a gel retardation assay that had previously been employed to examine the interaction of an IRE with its cytoplasmic binding protein (5−8).

MATERIALS AND METHODS
Preparation of detergent extracts
Detergent extracts were prepared in a buffer containing 25 mM Tris-HCl, pH 7.4, 40 mM KCl, 1% Triton X-100 from the following cell lines as previously described (8): a) human RD4 rhabdomyosarcoma cells; b) murine RAG renal carcinoma cells; c) Chinese hamster E36 cells; and d) 34 different human/mouse or human/Chinese hamster somatic cell hybrids derived from b) or c) that have previously been characterized (14). The somatic cell hybrids used were parallel thaws of frozen aliquots that had been extensively characterized cytogenetically and biochemically. All extracts were stored at −70°C.
Gel retardation assay
A [^{32}P]-labeled RNA probe was transcribed *in vitro* from a riboprobe plasmid ('TRS-1', Casey et al., manuscript in preparation) containing a synthetic oligonucleotide based upon the sequence of the regulatory region of the human TfR cDNA. This plasmid was linearized at a unique Bam HI site and transcribed using SP6 RNA polymerase. The labeled transcript, designated here 'TfR' (spec. act. approx. 1×10^8 cpm/μg RNA) was purified by repeated ethanol precipitation. The TfR probe contains 250 nucleotides that include three iron-responsive elements [previously designated elements 'B', 'C' and 'D' (4)]. Three different
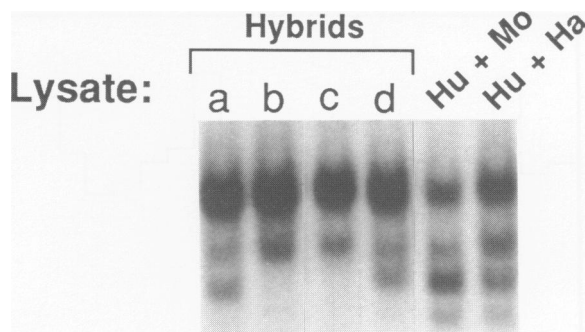
Figure 2. Human-rodent hybrid cell lines expressing human IRE-BP can be identified. Four different human-rodent hybrid cell lines (a−d) were analyzed for the presence of the human IRE-BP as described in the text. The IRE/IRE-BP complexes from these four cell lines and from the *in vitro* mixing experiment described in Figure 1 are shown. Note that extracts from hybrid cell lines a and d have both the human and rodent bands. The minor differences in mobility of the human complexes between lanes a and d were not reproducible and are likely explained by slight differences in the two acrylamide gels utilized in this experiment.

unlabeled transcripts were used as competitors at > 1000-fold molar excess: a) TfR; b) IRE [contains the human ferritin H-chain IRE as previously described (8)]; and c) 'unrelated stem-loop', a non-IRE containing transcript of similar length and secondary structure as b) with the RNA-sequence 5′AUAGCUAGCUAGCUAUGACAUAGCUAGCUAGCUAU3′ (Haile et al., manuscript in preparation).

Detergent extracts (13 μl) were incubated with 200,000 cpm of TfR probe at 22°C for 30 min. Subsequently, RNase T1 and heparin were added and the IRE/IRE-BP complexes were analyzed by non-denaturing gel electrophoresis as previously described (5,8), except that the electrophoresis was extended to 150 min at 165 V.

Chromosomal assignment
All 34 somatic cell hybrids analyzed were assigned a number code and blindly scored for the presence or absence of the human IRE-BP as determined by co-migration of the IRE/IRE-BP complex with the complex formed between the probe and the IRE-BP from human RD4 cell extracts. Adenylate kinase 1 (E.C. 2.7.4.3., AK-1) assays were performed as previously described (15). After the scoring, the code was revealed and the chromosomal assignment was made by discordance analysis according to published methods (16).

RESULTS
The IRE-binding protein recognizes specifically the IRE contained within the synthetic TfR probe
Extracts from human RD4 cells, murine RAG cells, and Chinese hamster E36 cells were analyzed by the gel retardation assay with the TfR probe. In each case, the resulting IRE/IRE-BP complexes migrate as a doublet (Figure 1). The complexes from RD4 cells migrate faster than the complexes from either RAG or E36 cells. The exact features of the complexes that give rise to the different electrophoretic mobilities are not yet known. However, the observation of distinct mobilities suggested that these differences might be used in chromosomal mapping. When extracts from human and rodent cells are mixed in a 1:1 ratio *in vitro* prior to the addition of probe, the human and the rodent IRE/IRE-BP complexes can be resolved (Figure 1). The bands seen represent specific IRE/IRP-BP
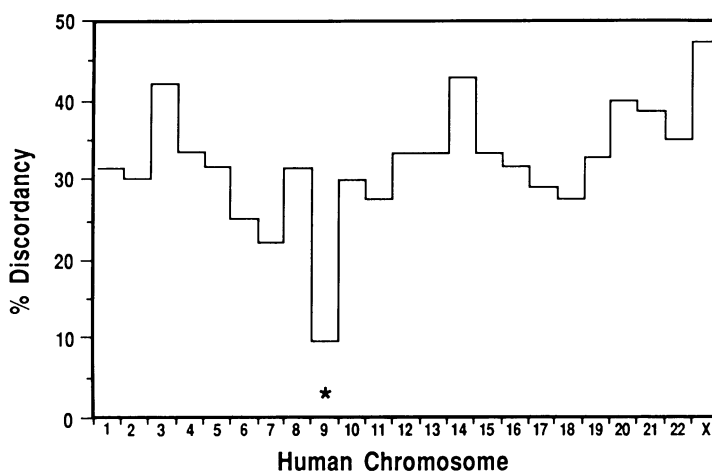
Figure 3. The gene encoding the IRE-binding protein is located on human chromosome 9. A panel of 34 different human/rodent somatic cell hybrids was analyzed by the gel retardation assay for the presence of the human IRE-BP. Each of the hybrids was scored as 'positive' (18/34), 'negative' (13/34) or 'probable' (3/34). The three hybrids that were scored as 'probable' were not included into the statistical analysis. The results were subjected to statistical discordance analysis as previously described (16). As shown, the presence of the human IRE-BP was only 9.7% discordant with human chromosome 9. All other human chromosomes had discordancies of >21.8%. Biochemical analysis of extracts from the same cells used for the gel retardation assay revealed only 3.1% discordancy with the chromosome 9 marker adenylate kinase 1 (indicated by the asterisk).

complexes, since their appearance can be completely abolished by pre-incubation of extracts with unlabeled TfR (Figure 1, left panel) or IRE transcripts (data not shown), but are not affected by competition with unlabeled beta-globin or unrelated stem-loop transcripts (data not shown).

The human IRE-BP can be expressed in human/rodent hybrid somatic cell lines
The ability to resolve the human from the rodent IRE/IRE-BP complexes in the *in vitro* mixing experiment led us to examine whether the human IRE-BP was expressed in some of the somatic cell hybrids. In Figure 2 are shown four representative cell lines of the 34 hybrids that were examined for the presence of the human IRE-BP complexes. The pattern of RNA-protein complexes observed for cell lines shown in lanes a and d were similar to the pattern seen in the *in vitro* mixing experiment whereas lanes b and c had only rodent complexes. Consequently, the human IRE-BP can be expressed and identified in extracts from some of the somatic cell hybrids. It is of note that, in cell lines scored as positive, both human complexes were present. This was particularly evident in longer exposures of the autoradiographs of the gel retardation assays.

The gene encoding the IRE-BP is located on human chromosome 9
We examined 34 different human/rodent somatic cell hybrid cell lines. Parallel thaws of all cell lines had previously been characterized by cytogenetic and biochemical analysis (14). Of these 34 cell lines, 11 were human/mouse hybrids and 23 were human/Chinese hamster hybrids. The human IRE/IRE-BP complexes were clearly detected in 13 of the 34 hybrids, 3 hybrids were scored as probably positive and not included into the statistical analysis while 18 of the 34 hybrids were scored as clearly negative. Figure 3 shows the results of a statistical discordance analysis based on these findings. The presence of the

human IRE/IRE-BP complexes is only 9.7% (3 out of 31) discordant with the presence of human chromosome 9 (as judged by previously established cytogenetic data). The discordance rate for the IRE-BP and the presence of the human chromosome 9 marker adenylate kinase 1 (AK-1) (17), was even lower (3.1%, 1 out of 31) (as judged by biochemical assay of the same cell extracts). In contrast, the discordance rates between the human IRE-BP and any other one of the human chromosomes ranged between 21.9% and 47.1%. The discordances between the IRE-BP and human chromosome 9 could be explained in several ways: First, it is not uncommon in isolated somatic hybrid cell lines that minute deletions or translocations escape the cytogenetic detection and give rise to false negative or false positive results. Second, parallel thaws of aliquots from the same cell line occasionally diverge in their cytogenetic patterns. All three discordances that were found had previously been cytogenetically negative for human chromosome 9, but showed the presence of the human IRE/IRE-BP complex. When the same cells used to examine IRE-BP activity were tested for AK-1 activity, they were all positive. We conclude that the gene that encodes the IRE-binding protein is located on human chromosome 9.

DISCUSSION

The assignment of the IRE-binding protein gene to human chromosome 9 excludes that this is the chromosome 6 gene for hereditary idiopathic hemochromatosis. The discordancy between the IRE-BP and human chromosome 6 was 25% (Figure 3). However, it remains entirely possible that a separate gene product from the hemochromatosis locus (6p21) influences the physiological function of the IRE-BP. To date, no other protein involved in human iron metabolism has been mapped to chromosome 9. Only a member of the extensive family of pseudogenes of the ferritin H-chain gene is found on chromosome 9 (18). Similarly, no known RNA-binding proteins, translation factors, or proteins involved in cellular stress responses have been located on chromosome 9 (19).

The method described in this study could prove to be particularly useful to establish the relationship between genetic regulatory factors (DNA- or RNA-binding proteins) and human genetic or acquired diseases. In addition, the identity of trans-acting factors with a known gene product such as an oncogene can be ruled out if the two genes can be localized to different chromosomes. The determination of the chromosomal localization of nucleic acid-binding proteins by this affinity mapping method is independent of the availability of cDNA probes or antibodies for the nucleic acid-binding protein of interest. The general application of this technique has only two requirements: a) a gel retardation assay which allows the specific detection of the human binding protein in a rodent background on the basis of a species-specific migration difference (as is the case with the IRE-BP) or a probe that is recognized only by the human binding protein; and b) the expression of the human binding protein in the somatic cell hybrid when the correct human chromosome is present. This latter requirement may limit the applicability of affinity mapping in cases of tissue-specific or developmental stage-specific binding factors which cannot even be induced to be expressed in these hybrid cell lines.

It should be noted that a human gene product mapped by this methodology may act in concert with other factors to form a protein-nucleic acid complex. These other factors could be encoded by the same human chromosome or be provided by the rodent genetic background of the hybrid. In the case of the human IRE-BP, purification by RNA affinity chromatography has indicated that this protein is a single polypeptide of 90 kilodaltons (20). Based on the results reported here, we have assigned the gene encoding the IRE-BP to human chromosome 9.

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