occupying two positions in the active site was derived by simulated annealing (with the slow-cool protocol) at 1000 K with the use of X-PLOR (*10*) with the flavin region omitted. Phases were further modified by a full-matrix Sayre refinement with SQUASH (*11*), followed by phase combination with the initial phases derived from simulated annealing. Amplitudes were weighted with SigmaA (*12*).

24. A mask representing the volumes inside the unit cell not occupied by protein was determined with a probe of radius 0.8 Å (*10*). Maps calculated with coefficients derived from the Fourier transform of the mask were contoured at 1 σ. The model of the active site in the "open" conformation was derived from the structure of the enzyme in complex with 2,4-DOHB. The side chain of Tyr^{222} was initially reoriented by manual modeling (*13*), followed by refinement of the atomic coordinates in the CHARMM22 force field in X-PLOR (*10*). Atoms within 5 Å of Tyr^{222} were restrained to their initial positions by application of a harmonic potential. Atoms outside this region were kept fixed during the minimization.

25. Supported by grants from NIH (GM 16429 to M.L.L., GM 20877 to D.P.B., GM 11106 to V.M., and Michigan Molecular Biophysics Training Program GM 08270 to B.A.P.), the University of Michigan (Rackham Graduate School Predoctoral Fellowship to B.A.P. and Rackham Graduate School Program to Support International Partnerships to D.P.B.), and the Australian Research Council (to B.E.). Coordinates have been submitted to the Protein Data Bank.

27 April 1994; accepted 2 August 1994

RNA Editing: Transfer of Genetic Information from gRNA to Precursor mRNA in Vitro

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RNA editing in the mitochondrion of *Trypanosoma brucei* extensively alters the adenosine triphosphate synthase (ATPase) subunit 6 precursor messenger RNA (pre-mRNA) by addition of 447 uridines and removal of 28 uridines. In vivo, the guide RNA gA6[14] is thought to specify the deletion of two uridines from the editing site closest to the 3′ end. In this study, an in vitro system was developed that accurately removed uridines from this editing site in synthetic ATPase 6 pre-mRNA when gA6[14] and ATP were added. Mutations in both the guide RNA and the pre-mRNA editing site suggest that base-pairing interactions control the number of uridines deleted in vitro. Thus, guide RNAs are required for RNA editing and for the transfer of genetic information to pre-mRNAs.

Mitochondrial pre-mRNAs in kinetoplastid protozoa have precise numbers of uridine residues inserted and deleted by RNA editing (k-RNA editing) (*1*). This possibly ancient (*2*) process produces mature mRNA sequences, often creating most of the coding information (*3*, *4*). Small (~60-nucleotide) guide RNAs (gRNAs) have been proposed to specify the sequences of edited transcripts by a combination of Watson-Crick and G:U base pairing (*5*). Most models for k-RNA editing postulate that the 5′ portion of a gRNA initially forms a short (anchor) duplex with the pre-mRNA that it edits and that subsequent editing of the pre-mRNA extends the complementarity of this duplex (*5–7*). Free uridine 5′-triphosphate (UTP) (*5*) or uridine residues at the 3′ end of gRNAs (*6*, *7*) may be the reservoir for the inserted and deleted uridines. As evidence exists in support of both of these possibilities (*7–13*), we developed an in vitro system to analyze the mechanism of k-RNA editing.

Editing of the 3′ end of *Trypanosoma brucei* ATPase 6 pre-mRNA appears to be directed by the gRNA gA6[14] (Fig. 1A) (*14*). The gRNA-mRNA chimeric molecules predicted by some models of RNA editing (*6*, *7*) are produced when these two RNAs are incubated with mitochondrial lysate (*9*). Characterization of these molecules showed that the pre-mRNA portion of several lacked uridine residues at the editing site closest to the 3′ end (ES1), a site from which uridines are deleted in vivo (*4*). Here, we investigated whether removal of these uridines required, and was specified by, the gRNA included in the in vitro incubation, using a procedure analogous to the assay of in vitro RNA editing employed in other systems (*15*). Our assay used dideoxythymidine (ddT) to halt reverse transcription at the adenosine immediately upstream of ES1 in a synthetic ATPase 6 transcript that was modified to distinguish it from endogenous mRNA (A6/TAG; see Fig. 1B) (*16*). A shorter product will be created if the A6/TAG transcript is processed correctly in vitro by gA6[14]. Subsequent treatment with a processive terminal transferase was used to add numerous deoxynucleotides to unextended primer and to products resulting from premature termination and "run-off" reverse transcription (which all terminate in 3′ hydroxyls). Molecules lengthened in such a way are prevented from entering high-percentage polyacrylamide gels, thereby allowing clear identification of extension products terminating in ddT.

Analysis of A6/TAG substrate after coincubation in mitochondrial lysate with an equimolar (Fig. 2, lane 8) or 10-fold molar excess (Fig. 2, lane 9) of synthetic gA6[14] showed two ddT-terminated primer extension products ("product" and "−2" in Fig. 2). Neither resulted from reverse transcription of endogenous RNAs, because neither was seen if A6/TAG was omitted (lane 5). Both products were present after terminal transferase treatment, which nearly completely shifted the unextended primer (compare lanes 1 to 3 with lanes 4 to 10 in Fig. 2, and compare lanes 1 to 3 with lanes 4 to 7 in Fig. 3B). Thus, both bands seen in Fig. 2, lanes 8 and 9, represent primer extension products that terminate in ddT. The upper band represents reverse tran-

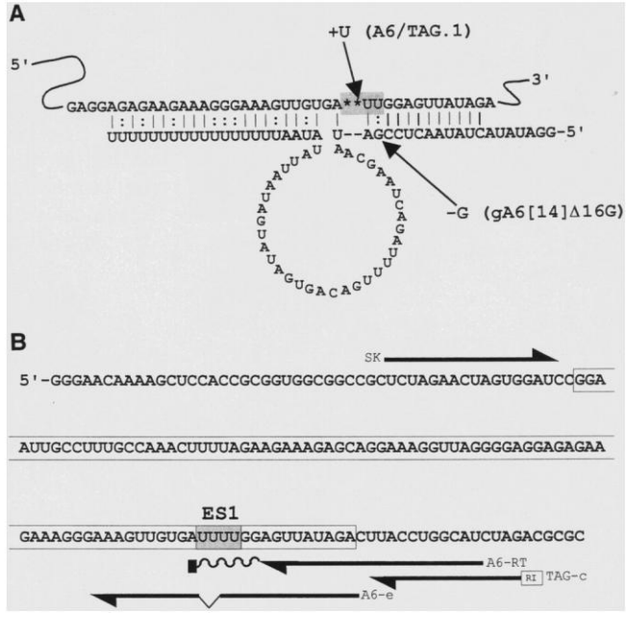

Fig. 1. (**A**) Possible A6/TAG–gA6[14] base pairing. Pre-mRNA is above and gA6[14] below. ES1 is shaded; uridines deleted in vivo are indicated by asterisks. A6/TAG and gA6[14] mutations are indicated by arrows. (**B**) A6/TAG transcript and oligonucleotides. ATPase 6 and heterologous sequence are boxed and unboxed, respectively. ES1 is shaded. Oligonucleotides are indicated as bars, with arrowheads representing 3′ ends. The ddT-terminated primer extension of A6-RT is indicated by a curved line and vertical bar. Oligonucleotide A6-e is complementary to molecules that have two uridines in ES1.

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scription of the input A6/TAG transcript. Production of the lower band requires mitochondrial lysate (lane 4). This product has the size expected if two uridines were deleted from the editing site, as compared with the RNA sequencing products of the same substrate (lanes 1 to 3). No −2 product was observed if the gRNA:substrate ratio was 0.1:1 (lane 7), which indicates that a threshold amount of gA6[14] is required for detection of processing. If the ratio of gRNA to substrate RNA was increased from 1:1 to 10:1, an increase in the amount of product was seen (lanes 8 and 9, respectively), which suggests that gA6[14] is the limiting component in the reaction. The endogenous pool of the gA6[14] gRNA in the extract could not support formation of the −2 product (lane 6), probably because its abundance relative to the substrate (0.0025:1) (*17*) was much lower than the 1:1 molar ratio required for exogenous gA6[14] to promote the smallest detectable amount of processing (lane 8). A noncognate gRNA (gCYB[558]) (*18*) did not result in formation of the −2 product (lane 10), which demonstrates that the required gRNA must be complementary to the appropriate region of the ATPase 6 transcript. Dependence on a specific gRNA is a fundamental property that is expected in authentic k-RNA editing (*5–7*).

As proposed for k-RNA editing (*5–7*, *12*, *13*), the sequence of the edited mRNA (represented by the −2 product) reflected the sequence of gA6[14] (see Fig. 1A). To test whether the number of uridines removed was determined by the sequence of our synthetic gRNA, we constructed a mutant gA6[14] RNA that lacked the guanosine at position 16 (gA6[14]Δ16G) and a mutant substrate RNA that contained an extra uridine at the editing site (A6/TAG.1) (Fig. 1A). If pre-mRNA–gRNA base-pairing interactions govern uridine deletion, then the mutant gRNA should cause a single uridine to be retained at the editing site regardless of the substrate processed; whereas the wild-type gRNA should specify the retention of two uridines at the editing site, independent of the substrate used (Fig. 3A). Indeed, two uridines remained and three were deleted from the editing site of the mutant pre-mRNA substrate when co-incubated with the wild-type gRNA (Fig. 3B, lane 4). Processing of this transcript with the mutant gRNA, however, left a single uridine and deleted four (Fig. 3B, lane 5). When the wild-type substrate (containing four uridines at the editing site) was co-incubated with the mutant gRNA, three uridines were deleted and one remained (lane 7), whereas two remained if it was co-incubated with the wild-type gRNA (Fig. 2A, lanes 8 and 9, and Fig. 3B, lane 6). These data indicate that information in the gRNA is transferred to the edited product in a way that strongly suggests that base-pairing interactions between the gRNA and the substrate at the processing site control editing.

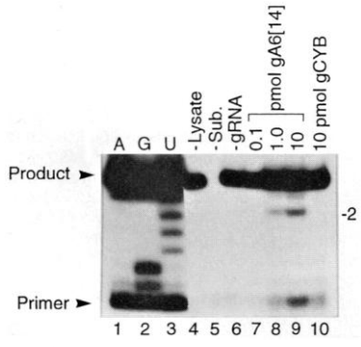

Fig. 2. A6/TAG primer extension analysis. In vitro reactions and primer extension were done as described (*28*). Lanes 1 to 3 represent RNA sequencing of A6/TAG with ddT (lane 1), with ddT and ddC (lane 2), or with ddT and ddA (lane 3). Samples in lanes 4 to 10 were subjected to in vitro incubation, primer extension, and terminal transferase treatment. Lanes 4 to 6 represent in vitro reactions in which mitochondrial lysate (lane 4), A6/TAG substrate (lane 5), or gA6[14] (lane 6) was omitted. Lanes 7 to 9 are derived from reactions that contained 0.1, 1.0, or 10.0 pmol gA6[14], respectively. Lane 10 is identical to lane 9, except that gA6[14] was replaced with gCYB[558] (*18*).

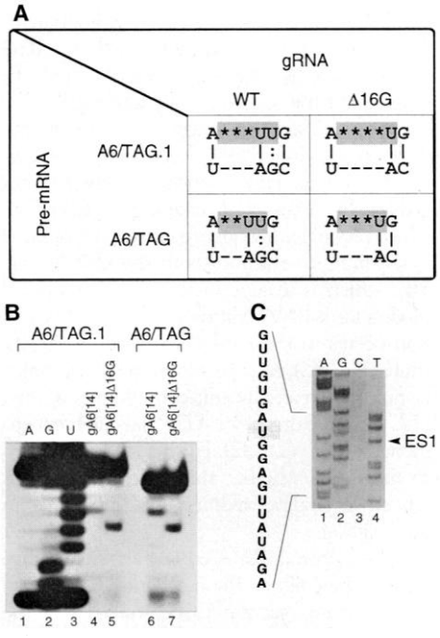

Fig. 3. (**A**) Pre-mRNA–gRNA base-pairing interactions. Potentially unpaired uridines, indicated by asterisks, are deleted. (**B**) Primer extension analysis. Lanes 1 to 3 are identical to lanes 1 to 3 in Fig. 2, except that A6/TAG.1 replaces A6/TAG. Lanes 4 to 7 show processing (*28*) of A6/TAG.1 with gA6[14] (lane 4) or with gA6Δ16G (lane 5), or of A6/TAG with gA6[14] (lane 6) or with gA6Δ16G (lane 7). Note the single-nucleotide difference in size between A6/TAG and A6/TAG.1 extension products. (**C**) Complementary DNA sequencing. The cDNA was made without amplification by standard procedures (*27*) with oligonucleotides TAG-c and SK after in vitro incubation as in (B), lane 7. Clones were screened for the −3 stop by colony PCR and sequenced. Shown are the sequencing reactions of clone 26.4 terminated with ddT (lane 1), with ddC (lane 2), with ddG (lane 3), and with ddA (lane 4).

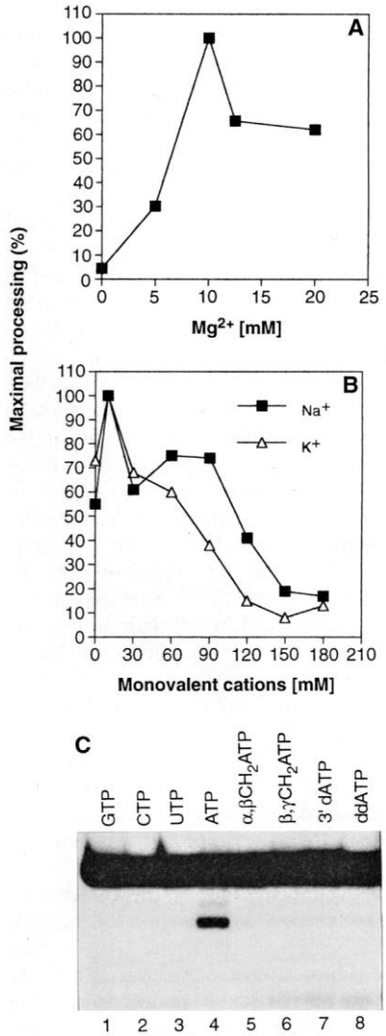

Fig. 4. Cofactor requirements. (**A**) Magnesium activity profile. Mitochondria representing 1×10^9 cells were lysed as described (*8*), and reactions were done as in Fig. 3B, lane 7, except that buffers contained either 0, 5, 10, 12.5, or 20 mM MgOAc. Processing efficiency was calculated by dividing counts per minute in the −3 band by the sum of counts per minute in −3 and unprocessed bands after excision from the gel and scintillation counting. Efficiencies were normalized to the reaction with the highest percentage of processing. (**B**) Monovalent cation activity profiles. Mitochondria representing 1×10^9 cells were lysed as described (*8*), and reactions were done as in Fig. 3B, lane 7, except that buffers contained KCl (triangles) or NaCl (squares) at 0, 10, 30, 60, 90, 120, 150, or 180 mM. Quantitation was done as in (A). (**C**) ATP dependence. Reactions were identical to those shown in Fig. 3B, lane 7, except that ATP was included (lane 4) or replaced with GTP, CTP, or UTP (lanes 1 to 3, respectively), or with α,β-methyleneadenosine 5′ triphosphate, β,γ-methyleneadenosine 5′ triphosphate, 3′ deoxy ATP, or dideoxy ATP (lanes 4 to 8, respectively).

To examine the entire sequence of the edited transcript and to exclude the possibility that the primer extension products arose from run-off reverse transcription of cleaved RNAs, we made complementary DNA (cDNA) using oligonucleotides complementary to heterologous sequences near the 5′ and 3′ ends of the A6/TAG substrate (SK and TAG-c, Fig. 1B) after in vitro reaction of A6/TAG with the mutant gRNA. Because a single uridine was left at the editing site when this gRNA-substrate pair was processed (Fig. 3B, lane 7), the RNA edited in vitro can be unequivocally differentiated from the edited ATPase 6 pre-mRNA produced in vivo. Five of 40 clones screened positive for the −3 deletion, and three of these were sequenced. All were identical and contained a single uridine (because of a −3 deletion) at ES1 (Fig. 3C). No other alterations were observed, even though gA6[14] can direct processing at 14 sites upstream of the one monitored. Thus, only a single cycle of editing may be possible under these in vitro conditions. Alternatively, because all but one of the sites required uridine addition, different in vitro conditions may be needed, as with the forward and reverse reactions catalyzed by self-splicing introns (*19*). Southern (DNA) blotting of reverse transcription–polymerase chain reaction (RT-PCR) products (amplified using SK and TAG-c) with an oligonucleotide that specifically recognizes edited RNA (A6-e; see Fig. 1B) also indicated that uridines were deleted from full-length A6/TAG in a gRNA and in a mitochondrial lysate–dependent fashion (*17*). We therefore conclude that the primer extension assay is faithfully tracking uridine deletion in the intact pre-mRNA, as is expected of the authentic k-RNA editing activity (*12*, *20*–*22*).

We examined some of the cofactor requirements for uridine deletion, again using the wild-type substrate and the mutant gRNA. The activity required Mg^{2+}, with optimal activity at a 10 mM concentration (Fig. 4A), but addition of monovalent cations was not required, and they inhibited the activity at moderate concentrations (Fig. 4B). Uridine deletion proceeded with ATP (Fig. 4C, lane 4) but not when nonhydrolyzable analogs about the α–β (lane 5) or β–γ (lane 6) phosphate bonds replaced ATP. The requirement for hydrolysis of α–β and β–γ phosphate bonds may indicate that ATP is needed for at least two distinct steps; 3′ deoxy ATP or dideoxy ATP (lanes 7 and 8, respectively) or other ribonucleotides, including UTP (lanes 1 to 3), could not substitute for ATP. Future studies are needed to determine if ATP is required for macromolecular complex assembly, "proofreading," catalysis, helicase activity, or some other function.

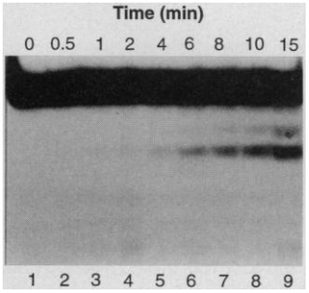

Fig. 5. Time course of uridine deletion. Ten-microliter aliquots were taken from a single reaction such as the one shown in Fig. 3B, lane 7, and processed as described (*28*). Lanes 1 to 9 represent aliquots taken at 0, 0.5, 1, 2, 4, 6, 8, 10, and 15 min, respectively.

To study the kinetics of uridine deletion, we took aliquots from a single in vitro reaction containing wild-type substrate and mutant gRNA after increasing lengths of time. Figure 5 shows that edited product was prominent after 15 min of incubation, with little or no lag time before product accumulation. In vitro pre-mRNA splicing systems have shown a latency of product formation due to assembly of a complex multicomponent ribonucleoprotein, the spliceosome (*23*). Rapid uridine deletion, therefore, may indicate that complex assembly, if necessary, is extremely rapid. Longer time courses (*17*) showed only a modest additional increase in product, which indicates that uridine deletion is active for only a short period (15 min) under the reaction conditions used.

Several observations suggest that the uridine deletion activity presented above represents k-RNA editing. The deletion activity requires gRNA (Fig. 2), as is the case in vivo (*24*). The edited pre-mRNA sequence reflects its potential base-pairing interactions with the gRNA (Fig. 3B), which is in agreement with all proposed models for k-RNA editing (*5*–*7*, *12*, *13*). Deletion occurs in the full-length pre-mRNA substrate (Fig. 3C), as expected from the existence of numerous partially edited transcripts in vivo (*12*, *20*) and from ^{32}P-UTP labeling experiments in vitro (*21*, *22*). Further study is needed to determine whether this in vitro process is related to uridine addition and represents in vivo editing.

The in vitro k-RNA editing activity that we describe here allows the mechanism of the uridine deletion reaction to be studied and the macromolecular components involved to be identified. Partial activities and macromolecular complexes that may be involved in k-RNA editing have previously been reported (*8*–*10*, *18*, *22*, *25*). Our results demonstrate that gRNAs are required for k-RNA editing and provide the source of genetic information. It remains to be determined whether gRNA–pre-mRNA chimeric molecules are intermediates in the reaction and whether the deleted uridines are donated to the U tail of the gRNA.

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16. Molecular biological manipulations followed established procedures (*27*). The product resulting from PCR of 3′A6UK (*9*) with oligonucleotides A6/TAG (5′-GCGCGTCTAGATGCCAGGTAAGTATTCTATAACTCCAAAATC-3′) and T3 (5′-ATTAACCCTCACTAAAG-3′) was gel purified and used as a template for run-off transcription with T3 RNA polymerase. A6-RT (5′-GATGCCAGGTAAGTATTCTATAACT-3′) was labeled by means of T4 polynucleotide kinase (Bethesda Research Laboratories, Baltimore, MD) [γ-^{32}P]ATP and used as a primer for reverse transcription. TAG-c (5′-CGCGGAATTCTAGATGCCAGGTAA-3′) and SK (5′-TCTAGAACTAGTGGATC-3′) were used to produce cDNA clones by standard procedures (*27*). PCR of gA6[14]NX (*18*) with oligonucleotides T7gA6wt (5′-GTAATACGACTCACTATAGGATATACTAT-3′) or T7gA6Δ16G (5′-GTAATACGACTCACTATAGGATATACTATATAACTCCATAACGAATC-3′) and T3 was used to produce templates for T7 transcription of gA6[14] or gA6Δ16G, respectively, after gel purification and restriction digestion with Dra I. A6/TAG.1 was produced as was A6/TAG, except that oligonucleotide A6/TAG.1 (5′-GCGCGTCTAGATGCCAGGTAAGTATTCTATAACTCCAAAAATC-3′) replaced oligonucleotide A6/TAG during PCR. The cytochrome b gRNA used in Fig. 3A has been previously described (*18*). Southern blots (*17*) were probed with oligonucleotide A6-e (5′-CTATAACTCCAATCACAACTTTC-3′).
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28. Thirty-microliter reactions were incubated for 40 min at 28°C and contained 10 μl of lysate (*8*) [~20 μg of protein (2 × 10^8 cell equivalents)] in 25 mM tris-HCl (pH 8.0), 10 mM MgOAc, 10 mM KCl, 3 mM ATP, 0.5 mM dithiothreitol, 0.16% Triton X-100, and 1.0 mM EDTA. All reactions contained 1 pmol of A6/TAG substrate and 10 pmol gA6[14] except as indicated in the figure legends. Reactions were stopped by the addition of 300 μl of NET-2[150 mM NaCl, 50 mM tris-HCl (pH 7.5), and 0.05% Nonidet P-40] and 1 μg of glycogen, extracted with phenol, and precipitated with two and one-half volumes of ethanol and one-tenth of a volume of 3.0 M NaOAc. Reverse transcriptions were done with radiolabeled A6-RT oligonucleotide at 48°C with Superscript II (Bethesda Research Laboratories) as described (*26*) but contained nucleotides at a final concentration of 0.4 mM dA, 0.4 mM dC, and 0.1 mM ddT. For RNA sequencing reactions, either 0.4 mM dA, 0.1 mM dC, 1.0 mM ddC, and 0.1 mM ddT, or 0.1 mM dA, 1.0 mM ddA, 0.4 mM dC, and 0.1 mM ddT was substituted as the nucleotide mix. Samples to be treated with terminal transferase were incubated with 0.5 μg of ribonuclease A for 30 min at 37°C, extracted with phenol, and precipitated with two and one-half volumes of ethanol and one-tenth of a volume of 3.0 M KOAc. Deoxynucleotidyl transferase reactions were incubated overnight at 37°C and contained 10 mM dTTP, 30 units of terminal deoxynucleotidyl transferase (TdT) (Bethesda Research Laboratories), 1× TdT buffer (Bethesda Research Laboratories), and primer extension products. Samples were run on gels containing 20% acrylamide, 8 M urea, and 1× TBE [8.9 mM tris-base, 8.9 mM boric acid, and 0.2 mM EDTA (pH 8.0)].
29. We thank P. Myler for suggesting the use of terminal transferase to distinguish dideoxy- and deoxy-terminated primer extension products and M. Ares, R. Braun, J. A. Steitz, D. Toczyski, and members of the Stuart laboratory for comments on the manuscript. Supported by NIH grant GM 42188 to K.S., who is also a Burroughs Wellcome Scholar of Molecular Parasitology.

25 May 1994; accepted 11 August 1994

Identification of the *ron* Gene Product as the Receptor for the Human Macrophage Stimulating Protein

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Macrophage-stimulating protein (MSP) is a member of the hepatocyte growth factor–scatter factor (HGF-SF) family. Labeled MSP bound to Madin-Darby canine kidney (MDCK) cells transfected with complementary DNA encoding Ron, a cell membrane protein tyrosine kinase. Cross-linking of ^{125}I-labeled MSP to transfected cells (MDCK-RE7 cells) and immunoprecipitation by antibodies to Ron revealed a 220-kilodalton complex, a size consistent with that of MSP (80 kilodaltons) cross-linked to the β chain of Ron (150 kilodaltons). The binding of ^{125}I-labeled MSP to MDCK-RE7 cells was inhibited by unlabeled MSP, but not by HGF-SF. MSP caused phosphorylation of the β chain of Ron and induced migration of MDCK-RE7 cells. These results establish the *ron* gene product as a specific cell-surface receptor for MSP.

Macrophage-stimulating protein (MSP) is an 80-kD disulfide-linked serum protein that induces the responsiveness of murine peritoneal resident macrophages to chemoattractants (*1*). MSP also acts directly as a chemoattractant for resident macrophages (*2*), causes shape change of macrophages (*1*), stimulates macrophage ingestion of complement-coated erythrocytes (*3*), and inhibits expression of inducible nitric oxide synthase mRNA in endotoxin- or cytokine-stimulated macrophages (*4*). MSP is synthesized in a biologically inactive form (pro-MSP) that can be cleaved to an active disulfide-linked heterodimer by specific serine proteases that include coagulation factors XIIa and XIa, plasma kallikrein (*5*), and also the glandular kallikreins [nerve growth factor–γ and epidermal growth factor binding protein (*6*)]. MSP is structurally related to HGF-SF (*7*, *8*).

Although MSP and HGF-SF both affect cell motility and morphology, the target cell specificities of the two proteins are different. MSP acts on resident macrophages (*1*, *2*), whereas HGF-SF affects epithelia and endothelia (*9*, *10*). These differences strongly suggest that the two proteins bind to distinct cell membrane receptors. The receptor for HGF-SF is the product of the proto-oncogene c-*met* (*11*). It is synthesized as a single chain precursor, which is subsequently cleaved to yield a disulfide-linked heterodimer (with a 40-kD α chain and an 150-kD β chain) with an intracellular protein tyrosine kinase domain (*12*). The receptor for MSP is not known. Recently, the *ron* gene, a member of the c-Met receptor family, was cloned from a human foreskin keratinocyte complementary DNA (cDNA) library (*13*). Comparison with c-Met suggests that the *ron* gene product is also a membrane-spanning disulfide-linked heterodimer with intracellular tyrosine kinase activity. Transcripts of the *ron* gene were found in a human transformed keratinocyte cell line and in normal human lung. The genes encoding human MSP (*3*) and Ron (*13*) are both located on the short arm of chromosome 3 (3p21), a region of frequent deletion or mutation in small cell lung and renal carcinoma (*14*). The genes encoding both HGF-SF and its c-Met receptor are located on chromosome 7q (*15*, *16*). The location of ligand and receptor on the same chromosome, and the structural similarities between MSP and HGF-SF, suggested that the ligand for Ron might be MSP (*13*).

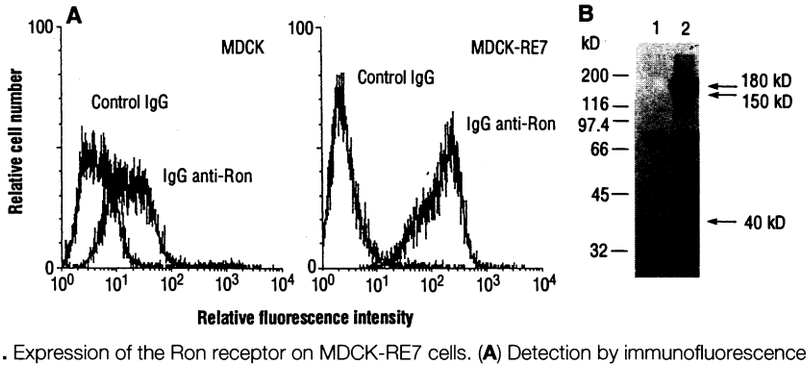

Fig. 1. Expression of the Ron receptor on MDCK-RE7 cells. (**A**) Detection by immunofluorescence with rabbit antibody to the extracellular domain of Ron (anti-Ron). (**B**) Immunoprecipitation of Ron from MDCK-RE7 cells with rabbit antibody to a COOH-terminal peptide of the Ron β chain (lane 2). Lane 1 shows the normal IgG control.

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