cDNA cloning and characterization of the protein encoded by RD, a gene located in the class III region of the human major histocompatibility complex

Jianhua CHENG, Kevin J. MACON and John E. VOLANAKIS*

Division of Clinical Immunology and Rheumatology, Department of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294, U.S.A.

The RD gene, initially defined in the mouse, has been mapped between the Bf and C4A genes in the human major histocompatibility complex class III region. Using the mouse cDNA as a probe, we isolated and sequenced human RD cDNA clones. The composite nucleotide sequence consisted of 1301 nucleotides, excluding a poly(A) tail at the 3′ end. It contained a single open reading frame encoding a polypeptide of 380 amino acid residues with a calculated molecular mass of 42274 Da. The most striking structural feature of the deduced amino acid sequence is a region consisting entirely of 24 tandem repeats of an Arg-Asp (or Glu) dipeptide. The human RD cDNA was expressed in *Escherichia coli* as a fusion protein with glutathione S-transferase and used to produce antisera in rabbits. Western blot analysis and immunoprecipitation of lysates of biosynthetically labelled HeLa cells indicated that RD is a 44 kDa nuclear protein.

INTRODUCTION

In humans, the class III region of the major histocompatibility complex (MHC) spans about 1100 kb of DNA and is located between the class I region at the telomeric end and the class II region at the centromeric end. The entire class III region has been cloned in overlapping cosmid and YAC clones [1–5] and the resulting gene map includes 36 loci. Among these are the genes encoding the complement proteins C4A, C4B, C2 and factor B, the cytochrome *P*-450 enzyme 21-hydroxylase, the major heat-shock protein HSP70, tumour necrosis factors α and β, and valyl-tRNA synthetase. The structure and function of the products of the remaining genes are currently unknown. However, the extremely high gene density observed in this region of chromosome 6 and the apparent association of certain human diseases with MHC class III haplotypes [6,7] has stimulated speculation about possible functional relationships among the proteins encoded by these genes [6].

The RD gene was initially identified in the mouse and mapped in the H-2S subregion of the MHC between the Bf and C4-Slp genes [8]. Nucleotide sequencing of cDNA clones predicted a 42 kDa polypeptide characterized by an unusual region consisting entirely of a basic (mostly Arg)/acidic (mostly Asp) dipeptide repeated tandemly 26 times. Codon usage indicated that this Arg-Asp (R-D) periodicity could not be explained by recent duplication events, suggesting that strong selective pressures are responsible for the conservation of the RD motif [8]. Using mouse RD cDNA as a probe, the human RD gene was also mapped in the class III MHC region and it was shown to be expressed in a variety of tissues [8]. Partial cDNA clones for human RD have been isolated and characterized [9]. In addition, genomic human RD clones also have been isolated and sequenced [10]. Alignment of the human genomic nucleotide sequence with the corresponding mouse cDNA sequence indicated that the human RD gene comprises 11 exons and spans approx. 6 kb of DNA. Several discrepancies exist between the two reported human RD nucleotide sequences [9,10].

In this paper we present the complete nucleotide sequence of the human RD cDNA, which was derived from two overlapping clones. In addition, we have raised antisera against recombinant RD polypeptide fragments and utilized them to identify and characterize the RD protein in HeLa cells as a 44 kDa nuclear constituent.

MATERIALS AND METHODS

Isolation and nucleotide sequencing of RD cDNA clones

Human RD cDNA clones were isolated from a T cell cDNA library in λgt11 (HL1016b; ClonTech, Palo Alto, CA, U.S.A.). The library was screened with the insert of the mouse RD cDNA clone WL623 [8], provided by Dr. M. Lévi-Strauss, Institut Pasteur, Paris, France. Several clones were isolated and two, HRD4.1 and HRD1.1, were used to derive the nucleotide sequence (see Figure 1). HRD4.1 extending from nt 200 to the poly(A) tail was sequenced in its entirety, while HRD1.1 was used to derive the nucleotide sequence of the 5′ region. Both strands were sequenced by the chain-termination method, using M13 mp18 and mp19 as cloning vectors and modified bacteriophage T7 polymerase [11] (Sequenase; United States Biochemical, Cleveland, OH, U.S.A.). To resolve compression bands, dITP/ddITP were substituted for dGTP/ddGTP in the sequencing reactions [12]. Nucleotide sequence data were analysed by using the Sequence Analysis Software Package of the University of Wisconsin Genetics Computer Group (Madison, WI, U.S.A.) and the MacVector Sequence Analysis Software (International Biotechnologies, Inc., New Haven, CT, U.S.A.).

Abbreviations used: MHC, major histocompatibility complex; GST, glutathione S-transferase; IPTG, isopropyl β-D-thiogalactoside; RT, room temperature; ORF, open reading frame; UTR, untranslated region; snRNP, small nuclear ribonucleoprotein.

* To whom correspondence and reprint requests should be addressed.

The nucleotide sequence data reported are available from GenBank under accession no. L03411.

PCR

Genomic DNA was isolated from the white blood cells of unrelated individuals as described [13]. Two oligonucleotide primers, 5′-AGTGATCGACTTCGAGAACT-3′ and 5′-TCAGTTAAGGTCACCTTGAC-3′, derived from the 5′ ends of exon 7 and intron 7 of the RD gene respectively [10], were used to amplify a 365 bp region of the gene. Amplification was performed by using 0.5 μg of genomic DNA and 2.5 units of DNA Taq polymerase (Amplitaq; Perkin–Elmer Cetus, Norwalk, CT, U.S.A.). The PCR was carried out for 30 cycles in a 100 μl reaction volume using a TempCycler (Coy Laboratory Products, Ann Arbor, MI, U.S.A.). Each cycle consisted of 1 min of denaturation at 94 °C, 45 s of annealing at 55 °C, and 1 min of extension at 72 °C, except for the last extension step which lasted 3 min. The PCR product was digested with *Eco*O109I and the overhangs were filled in. The resulting 271 bp fragment (nt 549–819) (Figure 1), containing the entire RD dipeptide repeat-encoding region, was cloned into the *Sma*I site of M13 mp18. From each amplified genomic DNA three independent M13 clones were isolated and sequenced.

Expression and purification of RD peptides

Two fragments from the coding region of the RD cDNA were expressed in *E. coli* as fusion polypeptides with glutathione S-transferase (GST). The first fragment (nt 210–1310; Figure 1), encoding all but the 41 N-terminal amino acid residues of the RD polypeptide, consisted of the 1.1 kb insert of the cDNA clone HRD4.1 and was obtained by *Eco*RI digestion of the λgt11 phage. The second fragment, a 372 bp long DNA fragment (nt 210–581, Figure 1) encoding amino acid residues 42–165, was obtained by *Eco*RI/*Hin*dIII double digestion of the HRD4.1 phage clone, followed by filling in the restriction enzymes overhangs. Both cDNA fragments were isolated by electrophoresis in low-temperature-melting agarose and were subcloned into the expression vector pGEX-2T (Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A.) in-frame with the GST gene, using the *Eco*RI and *Sma*I cloning sites for the first and second fragments, respectively. The orientation of the RD cDNA insert with respect to the GST gene was ascertained by using agarose gel electrophoresis of appropriate restriction digests. Competent *E. coli* cells, strain NM554, were transformed by the pGEX-2T-RD recombinants. Expression transformants were selected by inducing fusion protein expression with isopropyl β-D-thiogalactoside (IPTG) and analysing the cell lysates by SDS/PAGE [14]. Large-scale purification of fusion proteins was carried out from lysates of IPTG-induced cultures of transformants by using affinity chromatography on gluthathione–agarose as described [15]. For most experiments described here, RD polypeptides were cleaved from the GST carrier protein by digestion of the matrix-bound fusion polypeptide with thrombin as described [16].

Anti-RD sera

Rabbit anti-RD sera were raised by immunizing with the polypeptide RD-(42–380). For some experiments, antibodies against the RD-(42–380) or the RD-(42–165) polypeptide were purified from these antisera by affinity chromatography. The corresponding gel matrices were prepared by coupling approx. 3 mg of either fusion polypeptide to 5 ml of tresyl-activated agarose (Schleicher and Schuell, Keene, NH, U.S.A.), according to the manufacturer's instructions.

Cell culture

HeLa cells were grown in monolayer cultures in RPMI 1640 (Flow Laboratories, McLean, VA, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin and 10 μg/ml streptomycin at 37 °C in 5% CO_2.

Western blot analysis

Total cellular extracts were prepared by lysing HeLa cells in 60 mM Tris/HCl, 150 mM NaCl buffer, pH 7.4, containing 0.25% Nonidet P-40 and proteinase inhibitors (2 mM phenylmethanesulphonyl fluoride and 1 μg/ml each of leupeptin, aprotinin, pepstatin and chymostatin). Nuclei were isolated from HeLa cells and nuclear extracts prepared as described by Dignam et al. [17], except that all buffers contained the proteinase inhibitor cocktail described above. Extract proteins were fractionated by SDS/PAGE on 10% acrylamide gels and blotted on to nitrocellulose membranes electrophoretically as described [18]. Membrane strips were incubated at room temperature (RT) for 1 h in PBS, pH 7.4, containing 5% non-fat dried milk and 0.1% Tween-20 to block protein binding sites. Antibody-reactive protein bands were then detected by incubating the membrane strips at RT for 2 h in the same solution, containing a 1:500 dilution of pre-immunization or anti-RD rabbit serum, or 10 μg/ml affinity-purified antibodies. After washing, bound antibody was visualized by using a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL, U.S.A.) followed by alkaline phosphatase substrate (Sigma Chemical Co., St. Louis, MO, U.S.A.).

Biosynthetic labelling and immunoprecipitation

HeLa cells were grown in 10 cm diam. Petri dishes to about 75% confluency. The culture media were removed and the monolayer was washed with warm (37 °C) PBS, pH 7.4. Methionine-free media (4 ml) were added and after 1 h of incubation they were supplemented with 0.25 mCi of [^{35}S]methionine (DuPont–NEN Research Products, Boston, MA, U.S.A.). The cells were incubated at 37 °C in a CO_2 incubator for 18 h. The media were then removed, and the cells were washed three times with PBS, pH 7.4, and detached with a rubber policeman. Nuclear extracts were prepared as described [17] and incubated with 100 μl/ml Protein A–Sepharose 6B (Sigma) at 4 °C for 2 h. After centrifugation, the supernatant was made 10% glycerol and 0.5% Triton X-100 and incubated with 100 μl/ml Protein-A–Sepharose-bound anti-RD IgG. The antibody-coated beads were prepared by incubating Protein A–Sepharose with affinity-purified anti-RD-(42–165) IgG (10 μg of IgG/100 μl of beads) at 4 °C overnight and washing three times with PBS, pH 7.4, containing 0.1% Triton X-100. Control beads were prepared by incubating Protein A–Sepharose with normal rabbit IgG. The nuclear extract/beads mixture was rotated at 4 °C for 3 h. The beads were then collected by centrifugation and washed four times with PBS, pH 7.4, containing 0.1% Triton X-100, and once with 50 mM Tris/HCl, pH 6.8. Bound proteins were then extracted into SDS/PAGE sample buffer (62.5 mM Tris, pH 6.8, 5% 2-mercaptoethanol, 10% glycerol, 2% SDS, 0.005% Bromophenol Blue) by heating at 90 °C for 10 min, and subjected to SDS/PAGE on 10% acrylamide gels. After electrophoresis, the gels were soaked in 500 mM sodium salicylate for 30 min, dried by vacuum, and subjected to autoradiography at RT.

RESULTS

The nucleotide sequence of human RD cDNA, obtained by sequencing both strands of the two overlapping clones, HRD4.1 and HRD1.1, is shown in Figure 1. It consists of 1301 nt, which include a single 1143-nt-long open reading frame (ORF), a 86-nt-long 5′-untranslated region (UTR) and a 72-nt-long 3′-UTR, which is followed by a 17-nt-long poly(A) tail. A potential polyadenylation signal, AGUAAA, which deviates from the canonical AAUAAA signal but has been shown to be functional

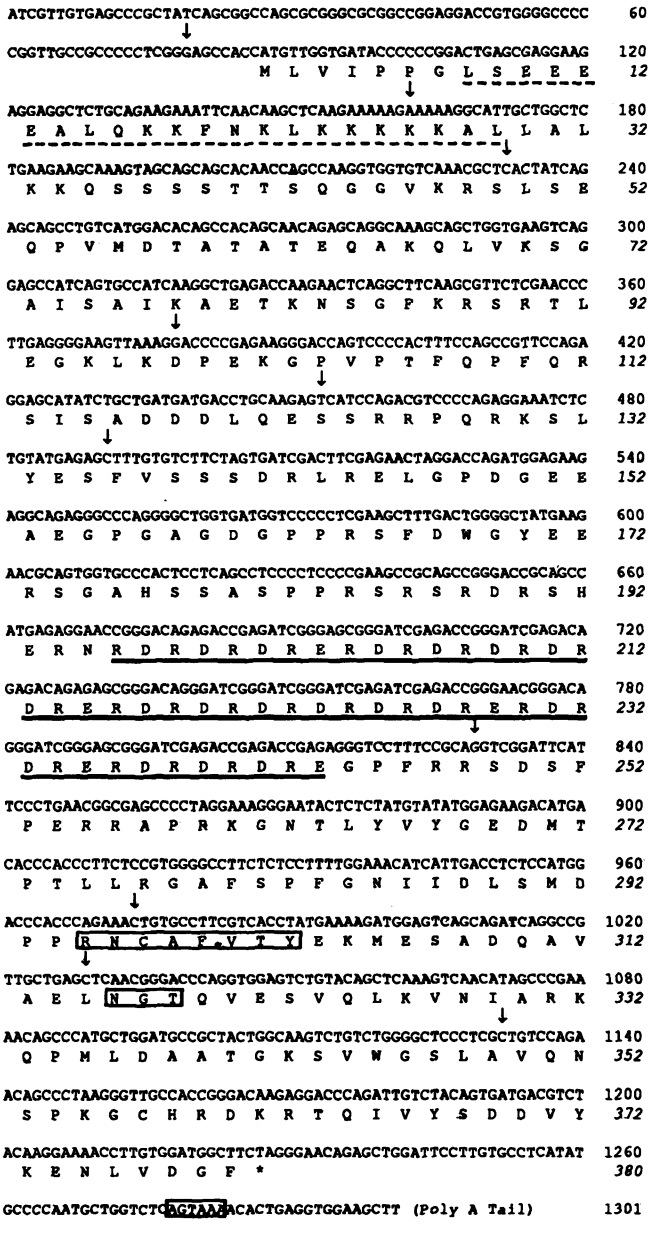

Figure 1 Nucleotide and predicted amino acid sequence of human RD cDNA

Nucleotide and amino acid residue numbers are on the right. Arrows mark the first nucleotides of exons 2–11. A leucine zipper motif is underlined by a broken line and the RD-repeat region by a solid line. Boxes show the putative RNA-binding motif [9], the potential N-glycosylation site, and the putative poly(A) signal, in that order. These nucleotide sequence data are available from GenBank under accession number L03411.

in vitro [19], is located 18 nt upstream of the poly(A) tail attachment site. The ORF, ending with a UAG termination codon, encodes a 380-amino-acid-residue polypeptide with a calculated molecular mass of 43274 Da. The putative AUG initiation codon lies within a sequence GCCACCAUGU, very similar to the consensus sequence motif of eukaryotic translation initiation sites, except for the presence of a pyrimidine at the 4+ position [20].

Comparison of the RD cDNA sequence reported here to that published previously [9] reveals two major differences. First, the present sequence is 216 nt longer at the 5′ end, while that reported by Surowy et al. [9] is 141 nt longer at the 3′ end. The difference at the 5′ end results in a longer ORF, encoding 55 additional amino acid residues. The deduced amino acid sequence of the extended N-terminal segment is very similar to that of the mouse RD [8]. In addition, as mentioned above, the AUG codon initiating the longer ORF lies in a sequence context that is highly suitable for translation initiation. Thus it appears likely that the cDNA clones isolated previously [9] were not full-length. The length difference at the 3′ end is perhaps more interesting, since both sets of cDNA clones include poly(A) tails. It seems possible that the observed difference results from the differential utilization of alternative poly(A) signals. This view is supported by the similarity between the extended 3′-UTR sequence reported by Surowy et al. [9] and that reported previously for the mouse RD [8]. However, the extended 3′-UTR of the human RD does not contain an additional typical poly(A) signal. Another difference between the two cDNA sequences is found in the codon for amino acid residue 174 (Figure 1), which is reported here as AGT for Ser as compared with the previously assigned ATG for Met [9]. It is not clear whether this difference is due to sequencing error or to genetic polymorphism. However, the nucleotide sequence of a genomic clone for RD [10] also has ATG (Ser) at the corresponding position.

Alignment of the cDNA sequence reported here with that of the previously characterized genomic RD clone [10] confirms the exon–intron organization of the RD gene that was deduced previously from comparison with the nucleotide sequence of the mouse cDNA (Figure 1). The present data also allow the definition of the boundaries of intron 1 of the RD gene, which was not previously possible. Inspection of the two nucleotide sequences indicates that intron 1 follows nt 78 of the cDNA sequence (Figure 1). The resulting first intron boundaries, ...CGGgtaagg... and ...cccctgactagG... (where exonic nucleotides are capitalized), are in good agreement with the consensus sequences for intron splicing [21]. Thus exon 1 encodes all but the last 8 nt of the 5′-UTR, which are encoded by exon 2 of the RD gene. Comparison of the ORF reported here with that proposed by Speiser and White [10] on the basis of the genomic sequence indicates that two codons of the latter sequence, GAC and GAT, both encoding Asp residues and corresponding to positions immediately following amino acid residues 116 and 265 (Figure 1), are not present in our cDNA clone. We have no explanation for this discrepancy. However, these codons are also absent from the partial cDNA sequence published by Surowy et al. [9]. Six additional nucleotide differences exist between the present sequence and that reported previously by Speiser and White [10]. In each case our sequence is in agreement with that published by Surowy et al. [9].

Inspection of the nucleotide sequence encoding the dipeptide repeat region indicates that it consists of four 36-nt-long imperfect tandomly repeated sequences. The presence of these nucleotide repeats, which display 81–89% identity with their consensus sequence, raises the possibility of unequal crossing-over events leading to length polymorphisms of the RD gene [22]. To

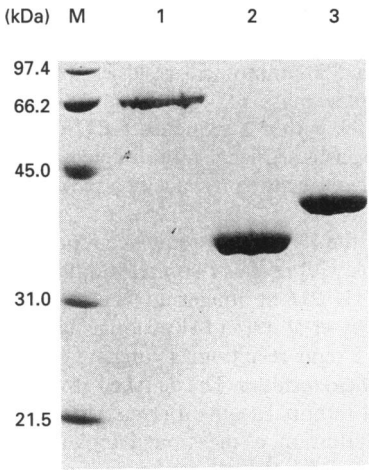

Figure 2 SDS/PAGE analysis of purified recombinant RD peptides

Lane M, size markers; lane 1, GST–RD-(42–380) fusion peptide; lane 2, isolated RD-(42–380) peptide, lane 3, GST–RD-(42–165) fusion peptide. The size of the markers in kDa is shown on the left.

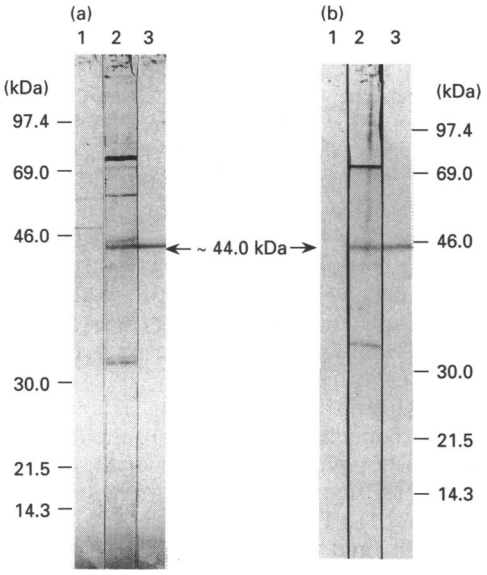

Figure 3 Western blot analysis of RD protein of HeLa cells

Total cellular (**a**) or nuclear (**b**) extracts of HeLa cells were subjected to SDS/PAGE and then blotted to nitrocellulose filters. Strips from each blot were incubated with control serum from rabbits before immunization (lanes 1), anti-RD serum (lanes 2), or affinity-purified anti-RD(42–165) antibodies (lanes 3). Bound antibodies were visualized by using alkaline phosphatase-conjugated goat anti-rabbit IgG, followed by alkaline phosphatase substrate. Positions and size in kDa of markers is shown. The 44 kDa polypeptide is indicated by arrows.

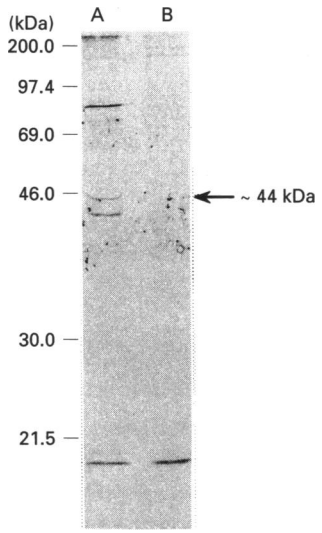

Figure 4 Immunoprecipitation analysis of RD protein

RD was precipitated from nuclear extracts prepared from ^{35}S-methionine-labelled HeLa cells by using affinity-purified anti-(RD42–165) antibodies (lane A). Lane B contains a mock immunoprecipitate obtained by using IgG purified from normal rabbit serum. Immunoprecipitates were subjected to SDS/PAGE followed by autoradiography. The positions and size in kDa of size markers is shown on the left.

investigate this possibility we amplified by PCR and sequenced this region from the genomic DNA of 12 unrelated individuals. At least 16 distinct MHC haplotypes were represented among these 12 individuals, including two haplotypes which were found to be associated with IgA deficiency and common variable immunodeficiency [7]. One of these haplotypes, characterized by deletion of the C4A gene, has also been found to be associated with systemic lupus erythematosus, insulin-dependent diabetes mellitus and coeliac disease [6]. None of the PCR fragments sequenced showed any length differences from the cDNA sequence shown in Figure 1.

Expression of the two RD cDNA fragments, nt 210–1301 and 210–581 (Figure 1), in *E. coli* by using the pGEX-2T vector resulted in the synthesis of GST–RD fusion peptides of the predicted size. As shown in Figure 2, the purified GST–RD-(42–380) fusion protein (lane 1) and the thrombin-cleaved and isolated RD-(42–380) peptide (lane 2) had apparent molecular masses of 66 and 36 kDa respectively, as compared with amino-acid-sequence-based estimates of 66 and 38.72 kDa respectively. The GST–RD-(42–165) fusion protein (lane 3) had an apparent molecular mass of 42 kDa, as compared with the estimated 40.8 kDa. Purified RD-(42–380) peptide was used to immunize rabbits. The antisera produced and affinity-purified antibodies thereof were utilized in the identification and characterization of human RD protein in HeLa cells.

Figure 3 shows Western blots of total cellular (Figure 3a) and nuclear (Figure 3b) extracts of HeLa cells developed with either unfractionated anti-RD-(42–380) serum (lanes 2) or with affinity-purified anti-RD-(42–165) antibodies (lanes 3). Lanes 1 show control blots reacted with serum obtained from the same rabbits before immunization. As shown, the anti-RD-(42–380) serum reacted with three major species of 73, 44 and 33 kDa. At least three additional, less prominent bands were present in the total cellular but not the nuclear extract. In contrast, the affinity-purified anti-RD-(42–165) antibodies reacted only with the 44 kDa species from either type of extract. It thus appears that the 44 kDa polypeptide, which is present in nuclei, represents the RD protein of HeLa cells. The identity of the additional bands reacting with the whole antiserum is not known. However, it seems possible that they represent cross-reactive proteins, since a number of nuclear proteins, including U1 small nuclear ribonucleoprotein (snRNP) 70 kDa, have basic–acidic dipeptide repeat regions [23,24], similar to although shorter than that present in RD protein. These charged regions are thought to be immunogenic [24] and thus likely to cross-react with RD protein.

This interpretation is consistent with the failure of antibodies against the RD-(42–165) peptide to recognize any of the additional protein bands, since this region of RD protein does not include the RD dipeptide repeats. If antibodies against the acidic–basic dipeptide repeat region are indeed responsible for the additional bands seen in the Western blot, the intensity of these bands may be a function of the relative abundance of the corresponding proteins in the HeLa cells extracts.

To further characterize the RD protein, we performed biosynthetic labelling experiments, followed by immunoprecipitation from nuclear extracts, SDS/PAGE and autoradiography. Affinity-purified anti-RD-(42–165) antibodies were used in these experiments, since they were shown to be specific for RD protein in the Western blotting analysis. However, as shown in Figure 4, immunoprecipitates obtained with these antibodies contained not only a 44 kDa species, which presumably represents RD protein, but also at least two additional polypeptides of about 84 and 42 kDa. Although the identity of these polypeptides is unknown, it seems possible that they constitute components of RD-containing macromolecular complexes.

DISCUSSION

The RD gene, named for an unusual region of its putative protein product characterized by alternating positively and negatively charged amino acid residues, mainly the repeating dipeptide Arg (R)–Asp (D), was initially identified as an MHC class III gene in mice. Here we report the full-length nucleotide sequence of the human RD cDNA, derived from overlapping clones. The reported sequence reconciles discrepancies between previously reported human RD sequences [9,10] and also allows for the description of the boundaries of the first intron of the gene (Figure 1).

The single ORF of the RD cDNA sequence encodes a 380-amino-acid-residue-long polypeptide with a calculated molecular mass of 43274 Da. Evidence for the expression of an RD protein of similar size was obtained for HeLa cells by Western blotting and immunoprecipitation experiments. Western blots, developed with antibodies against a region of the putative RD polypeptide not containing the repeating charged dipeptide and not exhibiting similarity with other proteins, identified a 44 kDa protein, present in total cellular and in nuclear extracts of HeLa cells (Figure 3). The nuclear localization of the RD protein was confirmed by indirect immunofluorescence (results not shown). The amino acid sequence similarity between the RD protein and the U1 snRNP 70 kDa protein and the presence of an snRNP-like domain in the RD protein [9] provide suggestive evidence for an association with snRNP particles or other macromolecular structures associated with spliceosomes [25,26]. The immunoprecipitation experiments indicating that the RD protein is most likely complexed with at least two other nuclear proteins is consistent with this hypothesis.

As mentioned previously [8], the most notable feature of the amino acid sequence of the RD protein is a 48-residue-long region starting at position 196 (Figure 1) and consisting exclusively of the tandemly repeated dipeptide of Arg and a negatively charged amino acid residue. Nineteen of the 24 negatively charged residues are Asp and the remaining five are Glu. As noted, [8,10], both the relatively poor conservation of codon usage and the lower similarity between mouse and human nucleotide sequences for this region than for the remaining cDNA indicate selective pressures for the conservation of the charge periodicity. This conclusion is supported by our failure to detect size polymorphism of this region of the RD gene, despite the presence of imperfect tandem nucleotide repeats. Admittedly the size of our sample was small; however, several MHC haplotypes were represented and two of them, which are associated with IgA deficiency and common variable immunodeficiency [7], are relatively common among Caucasians [27]. Furthermore, a similar search among 107 individuals indicated that only 3.3 % of the chromosomes carried genes with 22 or 23 repeats [28]. These considerations suggest an important functional role for the RD region of the protein.

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