Cloning of the Human Interferon-Related Developmental Regulator (IFRD1) Gene Coding for the PC4 Protein, a Member of a Novel Family of Developmentally Regulated Genes

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The rat PC4 gene had been initially isolated as a nerve growth factor-inducible sequence in PC12 cells. Although its function remains unknown, recently it has been shown that PC4 is necessary to muscle differentiation and that it might have a role in signal transduction. We report the isolation of the human homolog of the rat PC4 gene, renamed here IFRD1 (interferon-related developmental regulator 1). Several human IFRD1 clones were identified by searching the EST database using the rat IFRD1 (PC4) cDNA as a query. An EST clone containing the entire ORF was chosen for sequencing. Human IFRD1 presented a predicted protein product of 453 amino acids, highly conserved (90.2% identity) compared to the rat IFRD1 (PC4) protein sequences. The mapping assignment of human IFRD1 to chromosome 7q22–q31 was retrieved from the UniGene database maintained at NCBI. A comparison of human IFRD1 (PC4) protein to databases revealed 47% identity to the protein encoded by the human gene SKMc15, originally isolated from a chromosome 3-specific library. Therefore, SKMc15 is a gene related to IFRD1, being the second member of a novel family. We analyzed their expression during murine development, and we found that mouse IFRD1 appears more expressed in specific differentiating structures at midgestation, while mouse SKMc15 is highly expressed soon after gastrulation and in the hepatic primordium, suggesting an involvement in early hematopoiesis. © 1998 Academic Press

INTRODUCTION

With the aim of identifying genes involved in the process of neuronal differentiation, we have isolated in recent years several immediate-early genes activated in the rat cell line PC12 by nerve growth factor (NGF; Tirone and Shooter, 1989; Bradbury et al., 1991). This cell line, which is derived from a pheochromocytoma, differentiates into sympathetic neurons in the presence of NGF, thus representing a widely used in vitro model of the differentiative action of NGF on chromaffin cells in vivo (Greene and Tischler, 1976). We found that one of the genes isolated, PC4, presented a significant similarity with interferon-γ, a molecule known for its role in cellular differentiation (Tirone and Shooter, 1989). Such similarity was between the carboxy-terminal half of the PC4 cDNA-deduced protein and the whole interferon-γ protein, suggesting the existence of a functional domain within the PC4 protein. Furthermore, the mouse PC4 homolog (T1S7) was identified as a tetradecanoyl phorbol acetate-induced gene in mouse NIH3T3 cells (Varnum et al., 1989). Although PC4 function remains unknown, its expression was found to be regulated not only during neuronal differentiation in vitro and in vivo (Guardavaccaro et al., 1994; Iacopetti et al., 1996), but also during myoblast differentiation. PC4 is in fact expressed in the myoblast and in the differentiated myotube, with a transient decrease after the onset of differentiation. This aspect appears to have an important functional significance in muscle differentiation, as directly demonstrated by studies performed in the myoblast cell line C2C12. In fact, stable inhibition of PC4 expression, using antisense PC4 cDNA transfection or microinjection of anti-PC4 polyclonal antibodies, leads to impairment of myogenin and myosin gene expression as well as of morphological differentiation (Guardavaccaro et al., 1995). Microinjection data in myoblast pointed to the necessity of PC4 presence when the stimulus for differentiation is delivered, suggesting the involvement of PC4 in signal transduction (Guardavaccaro et al., 1995). This latter possibility was also implied by the observation that a fraction of the PC4 protein, which appears to be cytosolic (Guardavaccaro et al.,...
1994, 1995), translocates transiently to the inner side of the plasma membrane at the beginning of neuronal differentiation by NGF in the PC12 cell line (Guardavaccaro et al., 1994). As a whole, this evidence suggests that PC4 might play a modulatory role in development, during the transition of a determined cell lineage (e.g., muscle or neuron) to a committed phenotype.

Here we report the cloning, sequencing, and expression analysis in adult tissues of the human homolog of PC4. In compliance with the HGMW nomenclature rules, and with the properties observed so far for the protein product PC4, the gene has been renamed IFRD1 (interferon-related developmental regulator 1). In this study we analyzed in parallel, starting from early stages of development, the expression of IFRD1 and of the related gene SKMc15 (Latif et al., 1997), obtaining hypotheses on the functional role of these genes of the same family.

**MATeRIALS AND METHODS**

Sequence analysis and computer-assisted search of databases. Automated fluorescent DNA sequencing was performed using the ABI Prism 377 machines with both Dye Terminator and Dye Primer Cycle Sequencing chemistries on double-strand plasmid templates. Computer analysis of the sequences was performed with the Wisconsin Package version 8.1-UNIX (August 1995). Similarity searches were performed using BlastN, BlastP, and FastA algorithms against the GenBank (release 101, June 1997), EMBL (release 50.0, June 1997), Pin-Proélin (release 52.0, March 1997), and Swiss-Prot (release 34.0, November 1996) databases.

Northern analysis. Similar amounts of mRNA from different human tissues, blotted on a nylon filter, were hybridized with human IFRD1 cDNA 32P labeled with the hexamer primers procedure (Feinberg and Vogelstein, 1983). Hybridization was performed at 42°C for 18 h in 5× SSPE (3 M NaCl, 0.2 M NaH 2PO 4, 0.02 M EDTA), 2× Denhardt's solution (1% Ficoll 400, 1% polyvinylpyrrolidone, 1% BSA), 100 μg/ml sheared salmon sperm DNA, 2% SDS, 50% formamide. The mRNA amounts were controlled by hybridizing the filters to a β-actin probe.

Mice. C57B16 mice were mated between 9 and 10 PM Day 0.5 postcoitus was assumed to begin at midnight of the day of vaginal plugging. Pregnant female mice were killed by cervical dislocation, and embryos were staged according to Theiler (1989), collected in ice-cold PBS under a dissectors microscope (SV11; Carl Zeiss, Inc., Thornwood, NY), and fixed in 4% paraformaldehyde overnight.

In situ hybridization. In situ hybridization was carried out as described (Wilkinson and Green, 1990; Wilkinson, 1992), with minor modifications. Both paraffin-embedded and cryostat sections were analyzed. Dissected embryos were prefixed in 0.1 M sodium phosphate buffer (pH 7.3), 4% paraformaldehyde at 4°C overnight and embedded in Tissue-Tek (Miles Laboratories, Inc., Elkhart, IN). Cryosections (8 μm thick) were transferred onto gelatin/chromium (III) potassium sulfate-subbed and dried at room temperature. Before hybridization, slides were fixed. To obtain probes, mouse SKMc15 and mouse IFRD1 (the latter previously isolated by Varnum et al., 1989, named T1S7) were isolated from mouse embryo (E13.5–14.5) and adult mouse cDNA libraries. The isolates, identified by searching the EST database using T1S7 and human SKMc15 as a query, corresponded to mouse EST W29669, i.e., T1S7, and to mouse EST W67990, i.e., mouse SKMc15. Their identity was checked and confirmed by complete sequencing and by Southern analysis (not shown). The probes used were antisense and sense strands spanning the SKMc15 gene from nt 1 to 699 (referred to the sequence obtained) and the IFRD1 (T1S7) gene from nt 748 to 1496 (referred to the published sequence of T1S7; Varnum et al., 1989).

Transcription reactions with T7 or T3 polymerase (Ribobase Kit; Promega Biotechnologies, Madison, WI) were carried out in the presence of [γ-32P]ATP (Amersham Corp., Arlington Heights, IL). The template was then degraded with RNase-free DNase (Pharmacia), and the labeled RNA was purified through a Sephadex G-50 column and progressively degraded by random alkaline hydrolysis to improve access to RNA in situ. The probes were dissolved at a working concentration of 1×10 6 cpm/μl in the hybridization mix; 30 ml of the appropriate probe was added to each slide. Hybridization was carried out overnight at 55°C. The slides were then washed under stringent conditions (65°C, 2× SSC, 0.1% formamide) and treated with RNase. Autoradiography was performed with Kodak NT/B2 emulsion. Exposure times were between 15 and 25 days.

Negative controls using sense strands were tested on sections adjacent to those hybridized with the specific antisense strands to determine the basal background. These probes never gave a detectable signal.

**RESULTS**

Cloning and Sequencing of Human IFRD1

The rat IFRD1 (PC4) cDNA sequence was compared to EST databases (dBEst; Boguski et al., 1993) using the BlastN algorithm (Altschul et al., 1990). We detected 11 ESTs having significant homology to the query sequence, from Soares' infant brain, fetal liver–spleen, and placenta cDNA libraries as well as from other human muscle and testis cDNA libraries (October 1996). Sequence analysis was then carried out on cDNA clone 52803 (isolated from an infant brain cDNA library), corresponding to a human EST (H29134) homologous to rat IFRD1 with a P value in the BlastN output of 3.5×10 –88. Clone 52803 has a cDNA insert of about 1800 nt, containing the entire coding region. The first in-frame ATG is located 219 nt from the 5′ end of the clone and fulfills Kozak's criteria for an initiation codon (Kozak, 1984). Comparison of the complete sequence of clone 52803 with dBEst indicated the presence of several ESTs belonging to the same transcriptional unit and further extending the UTR 3′ end of about 450 nt. These ESTs were assembled to obtain the complete sequence of human IFRD1 cDNA, which has a total length of 2252 nt, with a 5′ UTR of 219 nt and a 3′ UTR of 676 nt (Fig. 1). The predicted protein product encoded by the ORF is 453 residues long (Fig. 1) with a calculated molecular weight of 50,685. A search for active sites using the program Prosite (Bairoch, 1990) showed three potential phosphorylation sites by cAMP-dependent protein kinase (Thr-9, Thr-127, Thr-373), several potential phosphorylation sites by casein kinase II, four by protein kinase C (Ser-95, Thr-353, Thr-373, Thr 416), and one by tyrosine kinase (Tyr-364). Also, potential N-myristoylation sites were detected (residues 13–25 and 105–110), which, however, according to previous experiments performed with rat IFRD1 (PC4) cDNA, might be not functional (Guardavaccaro et al., 1994).

IFRD1 and SKMc15, Two Members of a Novel Gene Family

Human and rat IFRD1 (PC4) sequences are highly homologous, with a significant identity at both the cDNA and the amino acid levels (86.3 and 90.2% iden-
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tity, respectively; Fig. 2). The mouse IFRD1 sequence, TIS7 (Varnum et al., 1989), is also shown to be highly conserved in relation to the human, with 87.1 and 90.6% identity between the nucleotide and the protein sequences, respectively (Fig. 2). The carboxy-terminal region of the human IFRD1 (PC4) protein shows significant similarity to the human interferon-γ (47.5% similarity, alignment not shown), as previously seen for the rat IFRD1 (PC4) protein (49% similarity; Tirone and Shooter, 1989). Interestingly, a comparison of the full human IFRD1 sequence with the GenBank database also detected a human cDNA called SKMc15 (Accession No. U09585) whose sequence, although different from human IFRD1, presented significant homology. This cDNA had been isolated from a human skeletal muscle cDNA library using as a probe an evolutionarily conserved sequence (LUCA14) from a chromosome 3-specific library (Latif et al., 1997; Wei et al., 1996). Percentage identities between nucleotide and protein sequences of human IFRD1 (PC4) and SKMc15 are respectively 48.1 and 47.6% (Fig. 2). Therefore, IFRD1 and SKMc15 appear to be different members of a family of genes. An interesting feature emerging by comparison between rat, mouse, and human IFRD1 and human SKMc15 sequences, which thus might be a distinguishing character of the family, is the existence of three domains with higher conservation (in SKMc15, aa 60–152, 285–341, 406–440). This is particularly evident in the carboxyl-terminal domain (Fig. 2), which is also the region of homology to the interferon-γ.

FIG. 1. Nucleotide and predicted amino acid sequence of human IFRD1 (PC4). The ATG start codon and the polyadenylation signal are double underlined. The translation of the longest ORF is shown above the nucleotide sequence with the one-letter amino acid code. The nucleotide sequence has been submitted to the EMBL database under Accession No. Y10313.
Tissue Expression of Human IRFD1 in Adult Tissues

The expression pattern of human IFRD1 was tested by hybridizing the 32P-labeled cDNA clone 52803 to a Northern blot containing poly(A)⁺ RNA samples from several human adult tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas). A 2.2-kb transcript, in agreement with the sequence length of IFRD1, was detected in several human tissues although at different levels of expression (Fig. 3). The human tissue expression of IFRD1 mRNA is very similar to that observed in rat (Tirone and Shooter, 1989), being very low in liver, lung, and kidney (see Fig. 2).
transcripts was quite different from that of SKMc15. In
the somites (Fig. 4G).

A signal was detected along the midhindbrain region and
embryonic expression was confined to the anterior and
atached structures and body organs, and at late
gestation ubiquitously. This general feature, thus, sug-
gested a complex regulation as well as different roles.

Expression of IFRD1 (TIS7) and SKMc15
During Development

To gain insight into a possible role for the SKMc15
and IFRD1 genes during embryonic development, we
studied their expression in mouse from gestation day
6.7 (E 6.7) to E 17. Fragments 700 and 750 bp long
were used as probes, respectively, for murine SKMc15
and murine IFRD1 (i.e., TIS7; see Materials and Meth-
ods). The sense strand of the same fragments did not
be detected in the mesenchyme cells and in the surround-

If the expression pattern of IFRD1 (TIS7) was more
complex, but restricted to a number of tissues and
organisms. These included the spinal ganglia, the devel-
oping kidney, the lung primordium, the olfactory and
respiratory neuroepithelium, the whole CNS with a
higher level in the developing telencephalon and dience-
phalon, and, finally, a restricted structure deriving
from the mandibular arch (Fig. 5F).

On the other hand, at E 10.5 SKMc15 was silent


during midgestation, the expression of SKMc15 and IFRD1 (TIS7) showed similar
patterns and complementary levels. In fact, while
SKMc15 was expressed at higher levels in early em-
byogenesis and then in the developing hepatic primor-
dium, IFRD1 (TIS7) was higher in differentiating
structures and body organs such as the developing
kidney, lung, CNS, spinal ganglia, and nasal neuroep-
ithelium, where SKMc15 was transcribed at lower
level. Finally, SKMc15 and IFRD1 (TIS7) expression
was studied at late gestation. At E 17, SKMc15 and
IFRD1 (TIS7) were widely distributed throughout all
the embryo, and their complementary expression pre-
viously observed at earlier stages was not evident.
However, several organs still expressed high levels of
both SKMc15 (Figs. 6A–6C) and IFRD1 (TIS7, Figs.
6E–6G), such as the liver, kidney, lung, spinal cord,
telencephalon, and salivary glands. In addition, as
shown at higher magnification, a relevant signal was
detected in the back muscles for both genes (Figs.
6D and 6H). In sum, the expression patterns of SKMc15
and IFRD1 (TIS7) indicate that these genes are tran-
scribed early, in gastrulating embryos mainly in ex-
traembryonic tissues, then at midgestation in re-
stricted structures and body organs, and at late
gestation ubiquitously. This general feature, thus, sug-
gests a complex regulation as well as different roles.

FIG. 3. Expression of IFRD1 in adult human tissues. The blots
contained 2 μg of poly(A)⁺ RNA/lane, obtained from eight different
human tissues. The filters were hybridized with the human IFRD1
(PC4) clone. Control hybridizations with a β-actin cDNA probe indi-
cated the presence of approximately equal amounts of RNA in all
lanes (data not shown).

Also Iacopetti et al., 1996) and maximal in pancreas,
skeletal muscle, and heart. Furthermore, the IFRD1
pattern of high expression in skeletal and cardiac muscle
and in pancreas is common also to SKM15 (Latif et
al., 1997). A lower and weaker band corresponding to a
smaller transcript of about 1.8 kb was also detected
(Fig. 3). Such signal did not correspond to SKMc15
mRNA, which has the same size of human IFRD1
mRNA (Latif et al., 1997). Excluding the possibility of
degradation, this might imply the presence of a tran-
script from another related gene.

Expression of IFRD1 (TIS7) and SKMc15
During Development

On the other hand, at E 10.5 SKMc15 was silent
throughout the embryo, except for a strong signal re-
stricted to the hepatic primordium (Figs. 5A and 5B)
and to the early circulating hematopoietic cells actively
generated in the differentiating liver (Fig. 5B and ar-
rowhead in 5B’). From this stage onward, SKMc15 was
detected at high levels in the hepatic primordium
(Figs. 5C and 6A–6C). A more detailed observation at
E 12.5 shows that, in addition to the liver, a lower
signal of SKMc15 was present in the kidney and lung
primordia as well as in the tongue and mandibular
structures deriving from the first branchial arch (Fig.
5C). Therefore, during early and midgestation, the ex-
pression of SKMc15 and IFRD1 (TIS7) showed similar
patterns and complementary levels. In fact, while
SKMc15 was expressed at higher levels in early em-
ymogenesis and then in the developing hepatic primor-
dium, IFRD1 (TIS7) was higher in differentiating
structures and body organs such as the developing
kidney, lung, CNS, spinal ganglia, and nasal neuroep-
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detected in the back muscles for both genes (Figs.
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and IFRD1 (TIS7) indicate that these genes are tran-
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traembryonic tissues, then at midgestation in re-
stricted structures and body organs, and at late
gestation ubiquitously. This general feature, thus, sug-
gests a complex regulation as well as different roles.
Among these, of particular interest is the highly restricted SKMc15 expression in the hematopoietic hepatic primordium as well as in early circulating megakaryoblastic precursors at 10.5 dpc, thus providing evidence of a possible role for SKMc15 in early embryonic hematopoiesis.

**FIG. 4.** SKMc15 and IFRD1 (TIS7) expression patterns in early murine embryogenesis. (A, C, E, and G) Sagittal section at E 6.7, 7.5, 8.2, and 9.5 hybridized with the SKMc15 probe. (B, D, F, and H) Sagittal sections at the same stages probed with the IFRD1 (TIS7) probe. The two genes were hybridized in adjacent sections of the same embryo except for the E 7.5 stage. (A' and C'–G') Bright fields of the corresponding sections. Abbreviations: a, amnios fold; ec, primitive ectoderm; ne, neuroectoderm; ch, chorion, me, mesendoderm; al, allantois; fg, foregut; so, somite; he, heart; fb, forebrain; hb, hindbrain.
FIG. 5. SKMc15 and IFRD1 (TIS7) expression patterns at early-midgestation. Adjacent sagittal (A, D, C, and F) and frontal (B and E) sections at E 10.5 (A, B, D, and E) and E 12.5 (C and F) probed with SKMc15 (A–C) and IFRD1 (TIS7) (D–F) genes. (A’–C’) Bright fields of the corresponding sections. Abbreviations as in Fig. 4 plus mb, midbrain; hp, hepatic primordium; sc, spinal cord; sg, spinal ganglia; st, stomach primordium; Te, telencephalon; Di, diencephalon; Mt, metencephalon; np, nasal pit; lu, lung; li, liver; ki, kidney. The arrows in D and E point to the IFRD1 (TIS7) signal in the spinal ganglia.
FIG. 6. SKMc15 and IFRD1 (TIS7) expression patterns at E 17. Sagittal (A, B, E, and F) and frontal (C and G) sections were hybridized with SKMc15 (A–C) and IFRD1 (TIS7) (E–G) probes, respectively. (D–H) Magnifications of back muscles probed with SKMc15 (D) and IFRD1 (TIS7) (H). (A‘–H‘) Bright fields of the corresponding sections. Abbreviations as in the previous figures, plus sa, salivary gland; mg, midgut; ag, adrenal gland; ty, thymus; cp, choroid plexus.
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Chromosomal Localization of PC4

Human EST clone 52803 is one of many EST sequences homologous to IFRD1 present in the Unigene database, at cluster 7879. STS 32586 (Genethon) and STS401 (Sanger Centre) have been generated from 3' EST sequences belonging to the same cluster. Radiation hybrid screening results for these STSs, generated by both Genethon and the Sanger Centre, indicated that the human IFRD1 (PC4) gene maps between markers D7S523 and D7S486, corresponding to 7q22-q31 (cytogenetic position 1.25–1.39 M, as by Chumakov et al., 1995).

DISCUSSION

The human cDNA whose cloning and sequencing is reported here encodes a protein having 90% amino acid identity to the rat and mouse IFRD1 (PC4 and TIS7) proteins. It can thus be assumed that this cDNA encodes human IFRD1. Sequence and tissue distribution of IFRD1 appear to be highly conserved between species, suggesting that the function of IFRD1 is conserved during evolution. The SKM15 gene, in virtue of its 47% identity to the IFRD1 sequences, appears to be the second member of a novel family of genes. Although the molecular function of IFRD1 and SKM15 is still unknown, hypotheses on their functional role are suggested from our analysis of expression. In the first place, both genes have high expression in skeletal and cardiac muscle of the adult human, as seen by Northern analysis. Furthermore, both genes are expressed in the embryonic skeletal muscle, attaining an appreciable level at the late gestation period. This fact, in line with the requirement for IFRD1 (PC4) observed in the process of muscle differentiation (Guardavaccaro et al., 1995), suggests that both proteins might share a common function(s), possibly encoded by the three regions of higher conservation. The same type of suggestion comes from the observation that during development both genes have qualitatively similar patterns, with early appearance in the embryo and extraembryonic tissues since gastrulation, followed by a common pattern of expression in restricted structures at midgestation (such as CNS, kidney, and lung primordia) or ubiquitous expression at late gestation. However, between the two genes there are strong and specific differences in the level of expression, since IFRD1 (TIS7) is expressed much more than SKM15 in differentiating tissues, such as nervous tissues, kidney, and lung, while SKM15 is highly expressed in the initial stages of embryogenesis and in the hepatic primordium. These findings agree with our previous analysis of IFRD1 (PC4) expression within the CNS, which showed that IFRD1 (PC4) was present not only in the ventricular zone of the neural tube at the moment of neuroblast proliferation, but also in the surrounding mantle zone where the differentiated neuron migrates (Iacopetti et al., 1996). Considering all together, it is plausible to imagine a role for IFRD1 in terminal differentiation of different cellular districts. This hypothesis is consistent with our previous observation of nuclear translocation of the IFRD1 (PC4) protein during neuronal differentiation (Guardavaccaro et al., 1994), which suggests a role for IFRD1 (PC4) in this process. Similarly, our present data are very suggestive of an involvement of SKM15 in the early differentiation of hematic precursors. It is interesting that SKM15, isolated as a putative tumour suppressor gene, has been excluded from being a such candidate, based on mutational analysis (Latif et al., 1997). Furthermore, we have observed that IFRD1 does not affect cellular proliferation (D.Guardavaccaro, unpublished results). It is therefore likely that SKM15 and IFRD1 are not impinging on cell cycle progression. Certainly further analyses, as well as functional comparisons between IFRD1 and SKM15, might help to assign a functional role to this gene family. Concerning the similarity between IFRD1 (PC4) and interferon-γ that we have previously observed (Tirone and Shooter, 1989), a comparison of interferon-γ proteins of different species with rat and human IFRD1 (PC4) still bespeaks common ancestry of these sequences (R. Doolittle, San Diego, pers. commun., December 1996). The interspecies conservation of the interferon protein, one of the fastest changing mammalian proteins, is, however, lower than observed for IFRD1, indicating different functional aspects and different positive selection for change. A comparison of the three-dimensional structures will thus be necessary for a better understanding of the evolutionary relation between IFRD1 and interferon-γ proteins. Concerning the chromosomal localization of IFRD1, which evidently differs from that of SKM15 (assigned to chromosome 3p21; Latif et al., 1997), we cannot at present exclude a functional correlation of IFRD1 with any of the diseases mapping to the IFRD1 locus. Further studies will therefore be necessary to ascertain whether IFRD1 is involved in diseases related to development and/or cellular differentiation.

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Note added in proof. After submission of the manuscript, a report appeared indicating that the locus linked to a severe genetic speech and language disorder colocalizes with the chromosomal region where IFRD1/PC4 gene maps, thus making IFRD1/PC4 a potential candidate responsible for such disease [Fisher, S. E., Vargha-Khadem, F., Watkins, K. E., Monaco, A. P., and Pembrey, M. E. (1998). Localisation of a gene implicated in a severe speech and language disorder. Nat. Genet. 18: 168–170.]
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