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Human cytoplasmic superoxide dismutase cDNA clone: A probe for studying the molecular biology of Down syndrome

(hybrid selection/restriction mapping/size classes of superoxide dismutase mRNA)

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ABSTRACT **The gene locus for human cytoplasmic superoxide dismutase (SOD-1; superoxide:superoxide oxidoreductase, EC 1.15.1.1) is located in or near a region of chromosome 21 known to be involved in Down syndrome. To approach the molecular biology of this genetic disease we have constructed a SOD-1 cDNA clone. Poly(A)-containing RNA enriched for human SOD-1 mRNA was isolated, used to synthesize double-stranded cDNA, and inserted into the endonuclease *Pst* I site of the plasmid pBR322. The chimeric molecules were used to transform *Escherichia coli*. Two clones containing SOD-1 cDNA inserts were identified by their ability to hybridize specifically with mRNA coding for SOD-1. Each of these clones carries a 650-base-pair insert, as was determined by restriction enzyme digestion and electron microscopic heteroduplex analysis. Hybridization of labeled cloned cDNA to RNA blots revealed two distinct SOD-1 mRNA classes of 500 and 700 nucleotides. The data suggest that both are polyadenylylated and are coded by chromosome 21.**

Superoxide dismutase (superoxide:superoxide oxidoreductase, EC 1.15.1.1) is the enzyme that catalyzes the removal of superoxide radicals, which are generated in a variety of biological oxidations (1). It provides a defense against oxygen toxicity and damage that may be caused to cells by carcinogenic hydrocarbons (1). The human Cu–Zn superoxide dismutase (SOD-1) is a dimeric protein composed of apparently identical noncovalently linked subunits, each with a molecular weight of 16,000–19,000 (2, 3). The locus for human cytoplasmic superoxide dismutase (*SOD-1*) was assigned to chromosome 21 (4).

About 1 in 600 newborn babies carries an extra chromosome 21, a condition technically known as trisomy 21 or Down syndrome (5, 6). This chromosome imbalance is a known cause of spontaneous abortion and mental retardation (5). In most cases, the patients with Down syndrome have karyotypes with 47 chromosomes (46 plus one additional 21). However, cases of Down syndrome in which only a portion of chromosome 21 is present in triplicate have enabled the localization of the "responsible" region to segment 21q22, the distal portion of the long arm (7–11). Although trisomy 21 was identified as a human genetic disease over 20 years ago (5), little is known about the mechanisms by which the extra chromosome or the extra chromosomal segment 21q22 results in reduced viability and abnormalities of morphogenesis and mental function. It is generally assumed that the extra chromosome or chromosomal segment codes for normal products and that the abnormalities found in Down syndrome are produced by an imbalance due to changes in gene dosage (12). Namely, the presence of additional genetic material in the cell will result in the production of commensurately increased amounts of the gene products coded by the extra chromosomal segment. Indeed, Down syndrome patients show an increase of about 50% in SOD-1 activity (13–15) due to a higher level of SOD-1 protein (16). However, it is not known whether this gene dosage phenomenon is a result of quantitative changes in the amount of SOD-1 mRNA.

In the past, most of the reports on Down syndrome involved family karyotyping and clinical studies of the effects of the disease on patients. It is only recently that recombinant DNA techniques have enabled us to approach the molecular biology of the chromosomal region involved and try to gain insight into the mechanism by which abnormal karyotypes result in abnormal phenotype.

In this report we describe the molecular cloning of *SOD-1* and its use to probe the SOD-1 mRNA.

MATERIALS AND METHODS

Materials. Reverse transcriptase (RNA-dependent DNA polymerase) from avian myeloblastosis virus was kindly supplied by Joseph Beard (Life Science, Gulfport, FL). Nuclease S1 and calf thymus terminal deoxynucleotidyltransferase were purchased from Miles and P-L Biochemicals, respectively. Restriction enzymes were purchased from New England BioLabs and were used according to the supplier's instructions. Nitrocellulose membrane filter (0.45-μm pore diameter) was obtained from Schleicher & Schuell. Labeled nucleotides were from the Radiochemical Centre (Amersham, England).

Cells. The trisomy 21 skin fibroblast culture is derived from a 1-month-old male and was obtained from the Human Genetic Cell Repository (Camden, NJ), culture no. GM-2504. The SV80 cells are a continuous line of simian virus 40-transformed human fibroblasts (17). The mouse–human hybrid cell line WAVR4dF9-4a (18) originating from the fusion of a mouse A9 cell and a human diploid fibroblast was obtained from F. Ruddle. It contains a full mouse genome plus human chromosome 21. The FS-11 cells are human fibroblasts from foreskins, established in our department by D. Gurari-Rotman (19).

Construction of cDNA Clones. A poly(A)-containing RNA fraction enriched for SOD-1 mRNA was isolated as described (3). Briefly, total poly(A)-containing RNA was prepared from SV80 cells and fractionated through a linear sucrose gradient. Fractions were assayed for mRNA encoding SOD-1 by *in vitro* translation and immunoprecipitation. The fraction containing SOD-1 mRNA (≈11S) was used for the synthesis of double-stranded (ds) DNA according to the procedure of Wickens *et al.* (20). ds cDNAs were introduced into the *Pst* I site of pBR322 by using the dG·dC tailing and hybridization protocol of Villa-Komaroff *et al.* (21). The chimeric molecules were used to transform *Escherichia coli* strain HB101 in the presence of $CaCl_2$ (22). Transformants that were tetracycline resistant were screened by *in situ* colony hybridization (23). Selection of

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Abbreviations: SOD-1, cytoplasmic superoxide dismutase; ds DNA, double-stranded DNA; bp, base pairs.

mRNA by hybridization was performed as described by Ricciardi *et al.* (24). Cell-free translation in rabbit reticulocyte lysate, immunoprecipitation, and $NaDodSO_4$/polyacrylamide gel electrophoresis were carried out as described (3). Plasmid DNA was isolated from chloramphenicol-amplified cultures by Triton X-100 lysis (25) and was purified on CsCl/ethidium bromide gradients. All experiments with recombinant plasmids were performed in accordance with the National Institutes of Health guidelines for recombinant DNA research.

Blot Hybridization. Hybridization of DNA with filter-bound RNA was carried out by using a variation of the method described by Thomas (26).

Heteroduplex and R-Loop Formation. Plasmid DNAs (0.06 μg) were digested with *Eco*RI, denatured for 10 min in 0.1 M NaOH/12 mM EDTA, and neutralized by adjusting the solution to 160 mM Tris·HCl, pH 8.5/400 mM $NaClO_4$. Formamide (recrystallized three times) was added to 50% (vol/vol) and renaturation was allowed to proceed at 37°C for 60 min. R-loops were prepared as described by Brack (27).

RESULTS

Construction of cDNA Clones and Translational Analysis. An earlier study (3) established that mRNA that codes for human SOD-1 sediments at 11 S in linear sucrose gradients and can be translated *in vitro* to an immunoprecipitable polypeptide of 19,000 M_r. This fractionation procedure was applied to obtain partially purified SOD-1 mRNA. A sucrose gradient fraction enriched for SOD-1 mRNA ($\approx$2 μg) was copied into ds cDNA and cloned in *E. coli* as described in *Materials and Methods*. Plasmid (200–500 ng) containing ds SOD-1 DNA was used for transformation and 20,000 transformed colonies with tetracycline resistant ampicillin-sensitive phenotype were obtained. Three thousand transformants were replated in duplicate and screened for recombinants by *in situ* hybridization (23), using [^{32}P]DNA complementary to the mRNA used for generating cloned material. Significant hybridization was detected in 300 of the 3,000 colonies analyzed. Seventy-five of the resultant positive colonies were selected on the basis of signal strength for further analysis by hybridization selection and cell-free translation (3, 24). Plasmid DNA, purified from individual colonies and immobilized onto nitrocellulose filters, was used to select mRNA by hybridization. The bound RNA was eluted and translated in a cell-free system. Translation products were immunoprecipitated by anti-SOD-1 serum and subjected to gel electrophoresis (Fig. 1). Out of the 75 clones selected for testing by the "mRNA-fishing translation" procedure, one was found to be positive. The eluted mRNA directed the synthesis of immunoprecipitable 19,000 M_r polypeptides (Fig. 1, lane 1) that comigrated with *in vivo* labeled SOD-1 (lane 7). Two other recombinant plasmids and the vector (lanes 2, 3, and 4) did not yield any immunoprecipitable proteins. Translation of mRNA from the enriched fraction, but before hybridization, showed a few protein bands after immunoprecipitation, one comigrating with SOD-1 (lane 5). By comparison, we estimate that $\approx$30% of the input SOD-1 mRNA was recovered in an active form after the hybridization elution procedure with the filter-bound positive plasmid. This plasmid was named pS61-10. From the 3,000 clones picked and replated, one more positive clone (designated pS58-13) was identified by colony hybridization using the cDNA insert of pS61-10 as a probe. Therefore, the overall frequency of SOD-1-positive clones was 0.06%.

Characterization of pS61-10. A restriction map of the pS61-10 cDNA insert is shown in Fig. 2. *Pst* I sites were reconstituted at both ends. The cDNA insert excised from the plasmid by *Pst* I is 650 bases in length (Fig. 2*A*). It was isolated and labeled with [α-^{32}P]dCTP by using DNA polymerase (large fragment),

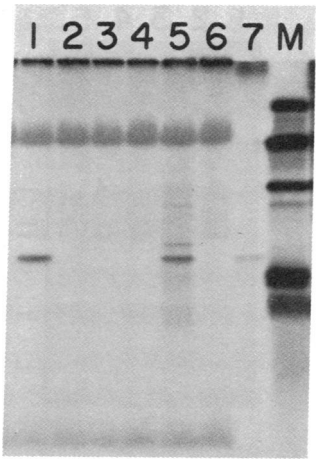

FIG. 1. Identification of SOD-1 cDNA clone by hybridization-selection and cell-free translation. Lanes 1–6, [^{35}S]cysteine-labeled polypeptides synthesized in reticulocyte cell-free systems, immunoprecipitated with anti-SOD-1 serum, and fractionated on a $NaDodSO_4$/15% polyacrylamide gel. Lanes 1–4, RNA selected by hybridization to immobilized plasmid DNA: lane 1, pS61-10; lane 2, pS61-9; lane 3, pS61-1; lane 4, pBR322. Lane 5, mRNA from 11S peak fraction of sucrose gradient; lane 6, no RNA added; lane 7, [^{3}H]leucine-labeled SOD-1 synthesized *in vivo* in the trisomy 21 fibroblasts GM-2504. Lane M, molecular weight markers: bovine serum albumin, 69,000; ovalbumin, 46,000; carbonic anhydrase, 30,000; lactoglobulin A, 18,367; cytochrome *c*, 12,300.

digested with a series of restriction endonucleases, and fractionated on 2% agarose gels. The insert contained cleavage sites for the enzymes *Mbo* II, *Taq* I, *Hae* III, *Alu* I, *Sau*3AI, *Hin*fI, and *Ava* II. Some of them were mapped by double digestion (Fig. 2*B*). The orientation of the insert with respect to the neighboring pBR322 sequences (shown in Fig. 2*B*) was determined by digesting pS61-10 with *Hha* I, eluting the 987-bp fragment, and subjecting it to secondary digestion by each of the following enzymes: *Alu* I, *Sau*3AI, *Hin*fI, *Ava* II, and *Taq* I (data not shown). Other enzymes did not cleave; these included: *Hae* II, *Hpa* II, *Bcl* I, *Hha* I, *Sac* I, *Sac* II, *Bgl* II, *Kpn* I, *Hpa* I, *Xho* I, *Sma* I, *Xba* I, *Bal* I, *Eco*RI, *Cla* I, *Hin*dIII, *Pvu* II, and *Sal* I.

The size and orientation of the insert cDNA were also analyzed by electron microscopic studies (Fig. 3). Plasmid DNAs of pS61-10 and pBR322 were made linear with *Eco*RI, for which there are no sites within the insert, and they reassociated to form heteroduplex molecules. All heteroduplexes contained a deletion loop of 650 $\pm$ 20 bp at $\approx$700 bp from one end of the duplex that maps at the *Pst* I insertion site (Fig. 3). This result was in good agreement with the length determined by agarose gel electrophoresis. R-loop structures were obtained by annealing linear pS61-10 DNA (cut with *Eco*RI) with poly(A)-containing SOD-1 mRNA isolated from SV80 cells and purified by fractionation on a sucrose gradient. The R-loops are located between short and long segments of duplex DNA and in some cases free tails are recognizable at the fork close to the short fragment (Fig. 3). We assume that the tails represent nonhybridized poly(A) sequences and thus the SOD-1 insert is in an opposite orientation to the β-lactamase gene.

Detection of SOD-1 mRNA in Human and Mouse Cells. Total cytoplasmic poly(A)-containing RNA was isolated from different cell cultures: FS-11 human fibroblasts, SV80 human transformed cell line, WAVR4dF9-4a mouse–human hybrid cell line, and A9 mouse cell line, which is the parent of the hybrid line. These RNAs were treated with formaldehyde and electrophoresed on 1.5% agarose gels containing 6% (vol/vol) form-

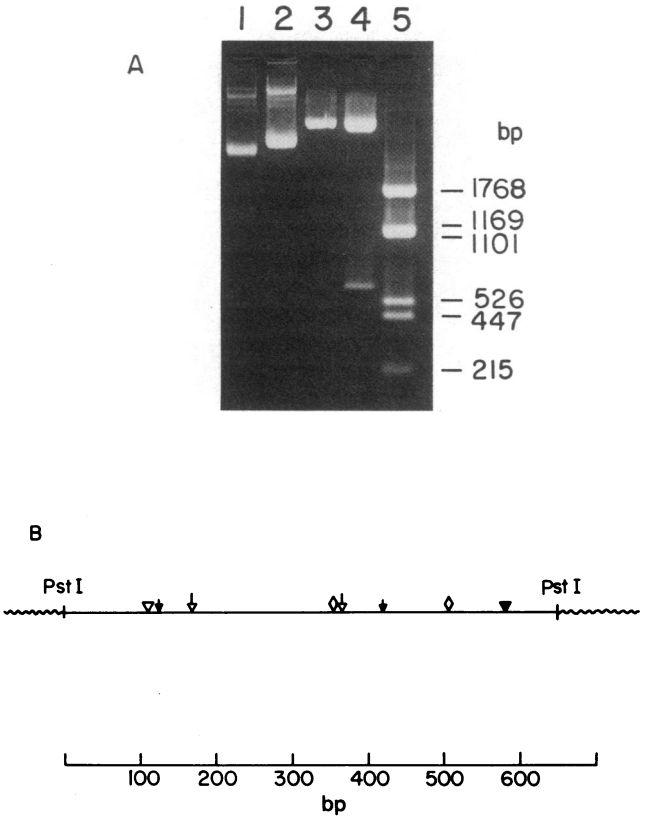

FIG. 2. Size analysis and restriction enzyme cleavage map of pS61-10 cDNA insert. (*A*) Plasmid DNA and restriction fragments were separated on 2% agarose gel. Lanes: 1, pBR322 DNA; 2, pS61-10 DNA; 3, pBR322 digested by *Pst* I; 4, pS61-10 digested by *Pst* I. The extra band that is 650 base pairs (bp) in length contains the SOD-1 cDNA. (*B*) Restriction map of pS61-10 cDNA insert. The 650-bp insert is drawn as a straight line flanked by pBR322 sequences as it is oriented in the plasmid (the *Eco*RI site is close to the right-hand side of the insert). *Pst* I sites correspond to the cutting sites on pBR322. ◇, *Sau*3AI; ⧫, *Hin*fI; ▼, *Ava* II; ▽, *Taq* I; ▼, *Alu* I.

aldehyde as described in *Materials and Methods* (26). The RNA was transferred to nitrocellulose filters and then hybridized to ^{32}P-labeled cloned SOD-1 cDNA (pS61-10). Two distinct size classes of SOD-1 RNA were detected in RNA extracted from the two human cells and the human–mouse hybrid line. These had molecular lengths (in nucleotides) of 700 ± 50 and 500 ± 50 (Fig. 4, lanes 1, 2, and 3). In RNA extracted from the mouse cell line A9 only the lower band was detected plus an additional very high molecular weight band (≈4,800 nucleotides; Fig. 4, lanes 4 and 5). The presence of the 700-nucleotide SOD-1 RNA in poly(A)-containing RNA extracted from the human–mouse hybrid cell line signified that this class is also coded by human chromosome 21, because this is the only human chromosome present in this hybrid line (18). On the other hand, the 500-nucleotide SOD-1 species is encoded by both the human and mouse chromosomes present in the hybrid line, as evident from the relatively higher amounts of this band (Fig. 4, lane 3). We have previously determined the size of human SOD-1 mRNA by *in vitro* translation of fractions along a CH_3HgOH agarose gel to be ≈420 nucleotides (3). Therefore we elected to assume that the 500 ± 50 nucleotide RNA species detected in Fig. 4 corresponds to the *in vitro* translatable mRNA. The nature and translatability of the larger SOD-1 RNA species require further investigation.

DISCUSSION

In this report, we describe the synthesis, cloning, and identification of a 650-bp cDNA bearing the sequence of human SOD-1 mRNA. Two size classes of human SOD-1 mRNA were identified, with ≈500 and ≈700 nucleotides. We therefore assume that the cDNA inserts of pS61-10 and pS58-13 that are relatively large (650 bp) contain most, if not all, of the mRNA sequences. The two cloned inserts seem similar because their restriction maps were identical and the heteroduplex analysis demonstrated a stable heteroduplex between them throughout the inserts.

As mentioned in the Introduction, cytogenetic analysis of mentally retarded patients has shown that trisomy of a small segment of chromosome 21—i.e., band 21q22—is sufficient to result in the phenotype of Down syndrome. More recently a normal SOD-1 activity was found in partial trisomy 21 (28), which suggests that translocation of the 21q22 chromosomal segment can reduce or even abolish SOD-1 expression. We believe that identification of the genes residing in this chromosomal segment, as well as detailed analysis of their organization, is of great importance in understanding the role differential gene expression (as in cases of gene dosage effects) plays in the phenotype differentiation. The gene locus of SOD-1 is

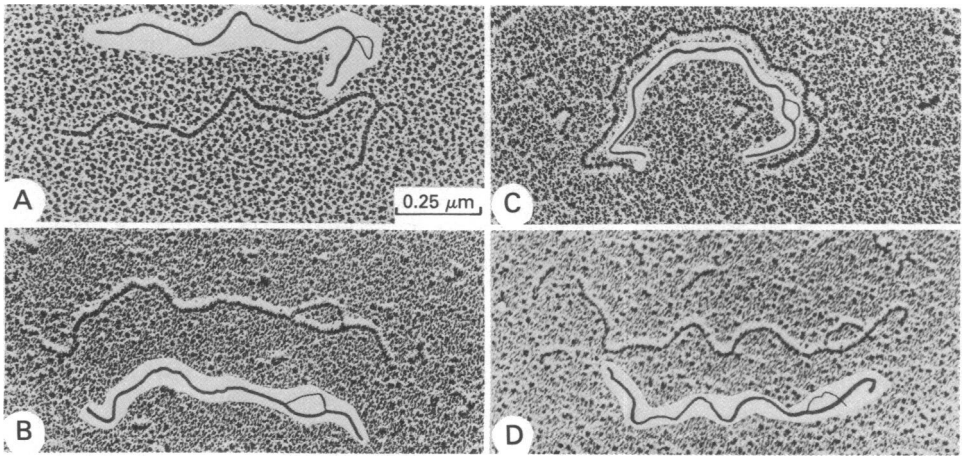

FIG. 3. Electron micrographs of heteroduplexes and R-loop structures. (*A* and *C*) Heteroduplex molecules of *Eco*RI-digested plasmids pS61-10 and pBR322 were prepared and visualized. The pBR322 arms provide the orientation of the cDNA. (*B* and *D*) R-loop structures of *Eco*RI-digested pS61-10 and cytoplasmic 11S poly(A)-containing RNA.

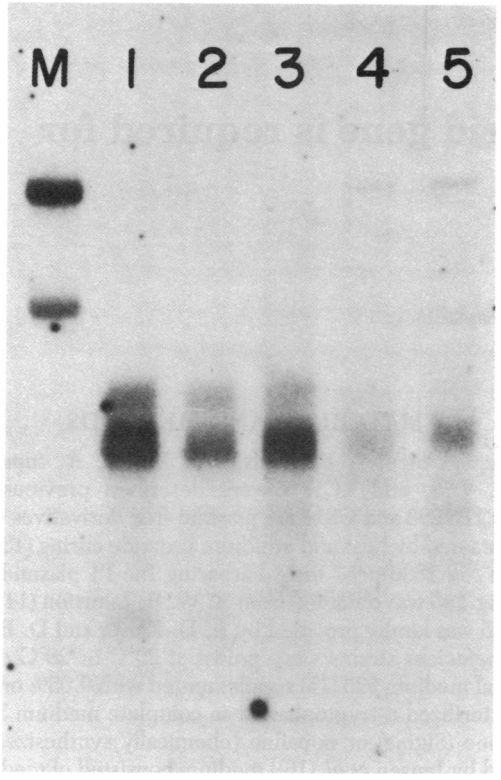

FIG. 4. Detection of human and mouse SOD-1 RNAs by blot hybridization. Unfractionated poly(A)-containing RNA (≈10 μg) was denatured, electrophoresed through a 1.5% agarose/formaldehyde gel, transferred to a nitrocellulose filter, and hybridized to nick-translated pS61-10 plasmid DNA. Lanes 1–4 contain poly(A)-containing RNA extracted from: 1, human transformed SV80 cell line; 2, FS-11 human fibroblasts; 3, WAVR4dF9-4a mouse–human hybrid cell line; 4, A9 mouse cell line (10 μg of RNA). Lane 5, 20 μg of A9 mouse cell line poly(A)-containing RNA; lane M, ^{32}P-labeled 28S and 18S rRNA markers.

located in the chromosomal band 21q22. It can therefore serve as a starting point for "chromosome walking" along the segment involved in Down phenotype and provide information about the linkage and expression of this gene and its neighbors.

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