must be contained within the proximal 7.6 kb of DNA 5′ to the AFP gene, as demonstrated recently in transgenic mouse strains where integrated AFP gene constructs exhibited *raf* regulation (*18*). The generation of transgenic mouse strains carrying different segments of the AFP gene regulatory domain (*19, 20*) should allow the fine mapping of the nucleotide sequences responsible for *raf* regulation, which will aid in the identification and eventual characterization of the *raf* gene product.

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Identification and Localization of Mutations at the Lesch-Nyhan Locus by Ribonuclease A Cleavage

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Many mutations leading to human disease are the result of single DNA base pair changes that cannot be identified by Southern analysis. This has prompted the development of alternative assays for point mutation detection. The recently described ribonuclease A cleavage procedure, with a polyuridylic acid–paper affinity chromatography step, has been used to identify the mutational lesions in the hypoxanthine phosphoribosyltransferase (HPRT) messenger RNAs of patients with Lesch-Nyhan syndrome. Distinctive ribonuclease A cleavage patterns were identified in messenger RNA from 5 of 14 Lesch-Nyhan patients who were chosen because no HPRT Southern or Northern blotting pattern changes had been found. This approach now allows HPRT mutation detection in 50 percent of the cases of Lesch-Nyhan syndrome. The polyuridylic acid–paper affinity procedure provides a general method for analysis of low abundance messenger RNAs.

THE LESCH-NYHAN (LN) SYNdrome is a severe, X-linked, recessive neurological disease that is the consequence of a defect in the hypoxanthine phosphoribosyltransferase (HPRT) gene, leading to HPRT enzyme deficiency (*1*). Analysis with HPRT complementary DNA (cDNA) probes has revealed that, in approximately 85% of LN cases, this gene appears normal by Southern and Northern blotting (*2*). This result suggests that most cases are the result of point mutations, or small DNA deletions or rearrangements. Among the LN cases with abnormal Southern blotting patterns, a heterogeneous collection of HPRT gene alterations has been observed. Together with the predominance of "small" lesions (below the resolution of Southern blotting), the heterogeneity of the mutations makes the characterization of HPRT deficiency at the nucleic acid level a difficult task.

Ribonuclease A (RNase A) cleavage has recently been employed to detect previously characterized β-globin mutations in genomic DNA (*3*) and c-Ki-*ras* variants in RNA from tumor cell lines (*4*). The RNase A cleavage assay is based on the fact that some single base mismatch sites in RNA hybrids with RNA or DNA will be cleaved by RNase A. A single RNA probe can be used to identify the presence of a base substitution, or a pair of overlapping probes can be used to unambiguously locate mutation sites. The precise requirements for susceptibility to RNase A attack are not yet clear, but it seems likely that 30 to 50% of possible single base mispairings will be cleaved (*3, 4*). Mismatches resulting from deletions, insertions, or rearrangements offer greater potential for RNase A cleavage because of more extensive single-stranded regions within the hybrids.

We have analyzed HPRT messenger RNAs (mRNAs) by RNase A cleavage. The mRNA can be examined with a single HPRT, antisense RNA probe generated from HPRT cDNA cloned into in vitro transcription vectors. This circumvents the difficulty of analyzing genomic DNA sequences, as the human HPRT coding sequences are distributed in nine exons spanning 44 kb of DNA (*5*). Preliminary RNase A cleavage experiments showed that HPRT mRNA, which represents 0.01% of the mRNA population (*6*), was difficult to detect in total cellular RNA preparations. To improve the sensitivity of the procedure we have used polyuridylic acid [poly(U)]–affinity paper (*7*) (Fig. 1). This "messenger affinity paper" (mAP) (Amersham) provides a solid support on which to recover RNA probe that is hybridized to polyadenylated HPRT mRNA, while nonpolyadenylated RNA, including unbound probe, is washed free. The HPRT mRNA can then be eluted from the mAP under conditions that will not denature the mRNA:RNA probe hybrids, before RNase A cleavage. This simple modification greatly increases the signal to noise ratio, presumably because it selects the polyadenylated fraction of RNA, containing the HPRT sequences, while simultaneously removing unbound radiolabeled probe that might contribute to background.

Analysis of RNA extracts from HeLa cells (which contain HPRT mRNA) and RJK 853, a human lymphoblastoid cell line with a total HPRT gene deletion ($HPRT^-$) (*2*), revealed that antisense HPRT RNA probes (Fig. 2A) are protected only when HPRT

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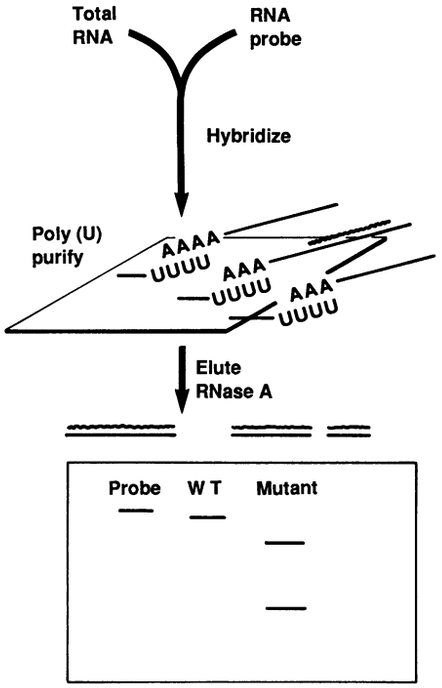

Fig. 1. Flow diagram for RNase A cleavage analysis with mAP purification. Total cellular RNA extracts were hybridized with radiolabeled RNA probe and the HPRT mRNA:probe hybrids were partially purified by passage through polyuridylic acid [poly(U)]–bound affinity paper. The hybrids were eluted, treated with RNase A to digest single-stranded regions and internal mismatch sites, and the fragments analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. A polyacrylamide gel is shown in schematic form to illustrate the appearance of the probe alone (Probe), probe hybridized to wild type HPRT mRNA (WT), and probe hybridized to a mutant HPRT mRNA that yields two fragments (Mutant).

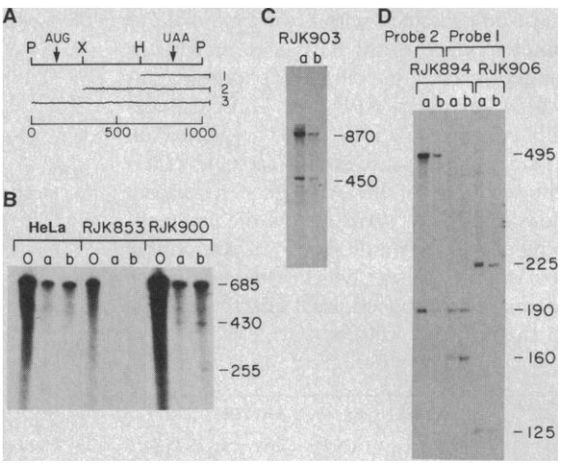

Fig. 2. RNase A cleavage patterns of normal (HeLa) and Lesch-Nyhan HPRT mRNAs. Total cellular RNA was analyzed according to (*9*). (**A**) Antisense RNA probes used in this study. Probes 1, 2, and 3 were generated after linearization of the HPRT template at the Hind III (H), Xho I (X), or immediately adjacent to the Pst I (P) site in the pTZ 19 polylinker with Eco RI, respectively. Probe 1 protected 350 nt of HPRT mRNA, probe 2 protected 685 nt, and probe 3 protected 955 nt. The samples in (B) were analyzed with probe 2; in (C) with probe 3; and in (D) with probes 1 and 2. (**B**) HeLa ($HPRT^+$); RJK 853, a total HPRT gene deletion LN case ($HPRT^-$) (*7*); and RJK 900, an LN patient with a known G to A transition ($HPRT_{KINSTON}$) (*8*). Samples were treated with RNase A, 0 μg/ml (0), 0.1 μg/ml (a), or 0.5 μg/ml (b). Fragment sizes are in nucleotides. (**C**) RJK 903 (GM 6804) (*7*), an LN case with a duplication of exons 2 and 3, treated with RNase A, 2 μg/ml (a), or 10 μg/ml (b). (**D**) RJK 894 and RJK 906, two LN patients with normal Southern and Northern blotting patterns, treated with RNase A, 2 μg/ml (a) or 10 μg/ml (b).

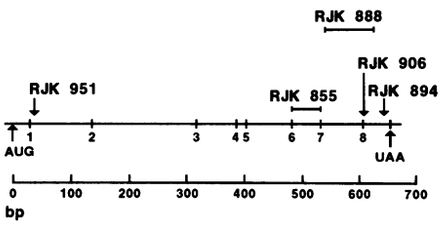

Fig. 3. Localization of mutation sites in Lesch-Nyhan HPRT mRNAs by RNase A cleavage. The sites of three possible point mutations (RJKs 894, 906, and 951) are indicated by arrows. RJK 855 and RJK 888 are deletions of approximately 40 and 85 nt, respectively. The RJK 855 deletion corresponds to exon 7 and the RJK 888 deletion terminates within exons 8 and 9. The 3′ end of each of the 9 HPRT exons is indicated.

mRNA is present (that is, in HeLa cells), confirming the specificity of the procedure (Fig. 2B). When RNA from an LN patient (RJK 900) with a known G to A transition in the HPRT gene ($HPRT_{KINSTON}$) was examined (*8*) two distinctive cleavage products were observed. The two fragments are the expected sizes from cleavage of the C:A mismatch in the RNA:RNA hybrid [430 and 255 nucleotides (nt)] and, from the intensity of the bands, it appears that approximately 5% of the fully protected HPRT probe was converted to the smaller fragments (Fig. 2B). This is a low efficiency of cleavage, compared with 100% cleavage of U:C, A:G, or A:A mismatches reported in RNA:RNA duplexes (*4*) or 50 to 100% cleavage of C:A mismatches in RNA:DNA hybrids (*3*), and may be a consequence of the sequence context of the mismatch. Cleavage efficiency in this case was not improved when we increased the amount of RNase A (up to 10 μg/ml). Approximately 30% internal cleavage was observed from analysis of RNA from $HPRT_{LONDON}$ (*8*), a partial HPRT deficiency patient with a C to T transition that generates a G:U mismatch. RNA from RJK 903, an LN patient with an exon 2,3 duplication in the HPRT gene and a correspondingly elongated HPRT mRNA (*2*), protected two fragments. The large fragment (870 nt) corresponds to protection from exons 2 through 9 and the smaller (450 nt) fragment corresponds to protection from exons 1 through 3 (Fig. 2C).

Distinctive RNase A cleavage patterns were also observed in 5 of 14 LN patients who had been previously classified as having no changes by Southern or Northern analysis. In all five cases 100% of the probe was cleaved. The sum of the length of the cleavage products equaled the length of the fully protected probe in three cases (RJK 894, 906, and 951), but revealed approximately 40- and 85-nt deletions in the mRNA of two others (RJK 855 and 888). Overlapping probes localized the lesions with respect to the ends of the HPRT mRNA. Examples of the mutant cleavage patterns are shown in Fig. 2D and the location of the mutation sites is shown in Fig. 3.

One of the deletion cases, RJK 855, seems to lack exon 7 in the HPRT mRNA and, thus, is a candidate for a gene splicing mutation. The deletion observed in RJK 888 terminates within exons 8 and 9 and is likely to be the result of a small DNA deletion including parts of exons 8 and 9 and the intervening sequences. RJKs 894, 906, and 951 may each be point mutations, as indicated by the size of the cleavage products. They may also be deletions or rearrangements of less than 5 base pairs, which would be consistent with the nearly 100% conversion of the full length probe to smaller fragments, in contrast to the limited cleavage observed in the two known point mutation cases.

No RNase A cleavage was observed in samples representing a total of 17 normal ($HPRT^+$) X chromosomes. Thus, it is likely that the different cleavage sites in the five LN patients represent the true mutation sites and are not DNA sequence polymorphisms that lead to redundant codon changes or silent amino acid changes. The absence of detectable RNase A cleavage in RNA from 9 of the 14 LN patients analyzed is also informative, and the simplest explanation for these cases is that they contain single base substitutions that do not result in base mismatches susceptible to RNase A cleavage. Therefore, the HPRT inactivity in each of these patients is not the result of gene deletions, rearrangements, or RNA splicing mutations.

We now favor RNase A cleavage analysis as a primary method to screen for altered nucleic acid sequences at expressed gene loci. In addition to the many gene alter-

ations that are uniquely detectable by RNase A cleavage, most changes that can be found by Southern or Northern blotting are easily identified, which brings the total fraction of LN mutations that can be diagnosed at the nucleic acid level to 50% (5/14 plus 15%).

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Isotopic labeling was achieved with 100 μCi of [^{32}P]guanosine triphosphate (650 Ci/mmol, diluted from 3000 Ci/mmol) (Amersham). Total cellular RNA (200 μg) [J. M. Chirgwin, A. E. Przybyla, R. J. MacDonald, W. J. Rutter, *Biochemistry* **18**, 5294 (1979)] from HeLa or lymphoblast cell lines transformed with Epstein-Barr virus was mixed with 5 × 10^6 cpm of probe in 200 μl of 80% formamide, 400 m*M* NaCl, 40 m*M* Pipes, *p*H 6.7, heated to 85°C for 5 minutes, and hybridized overnight at 65°C. The mixture was cooled to 25°C, diluted with 100 μl of 0.5*M* NaCl, and slowly applied to a 0.4-cm^2 piece of mAP that had been soaked in 0.5*M* NaCl for 5 minutes. The paper was washed four times for 15 minutes each in 0.5*M* NaCl at 25°C and heated to 65°C for 10 minutes in 180 μl of water to elute the HPRT mRNA:probe hybrid. The mAP was removed, the eluate chilled on ice, and 20 μl of 2.5*M* NaCl added. 100 μl of RNase A was added in 200 m*M* NaCl, 100 m*M* LiCl, 30 m*M* tris, *p*H 7.5, and 3 m*M* EDTA, and the samples incubated for 30 minutes at 25°C. Pretreatment of RNase A and the subsequent analysis of the RNase A cleavage products were according to Winter *et al.* (*4*). One half of the original reaction mixture (equivalent to 100 μg of total cellular RNA) was loaded in each lane. Autoradiographic exposures range from 12 to 16 hours, with one intensifying screen (Cronex) and Kodak X-AR5 x-ray film at −70°C. ^{32}P–end labeled Hae III-digested ΦX174 DNA fragments were employed as molecular size markers on the polyacrylamide gels.

10. R.A.G. is a research fellow and C.T.C. is an investigator of the Howard Hughes Medical Institute. We also thank J. T. Stout and P. I. Patel for valuable discussions and M. F. Browner for bringing mAP to our attention.

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Expression and Processing of the AIDS Virus Reverse Transcriptase in *Escherichia coli*

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The ability to express the genes of pathogenic human viruses, such as the acquired immune deficiency syndrome (AIDS) virus (also called human immunodeficiency virus) in bacterial cells affords the opportunity to study proteins that are ordinarily difficult or inconvenient to obtain in amounts sufficient for detailed analysis. A segment of the AIDS virus *pol* gene was expressed in *Escherichia coli*. Expression resulted in the appearance of reverse transcriptase activity in the bacterial cell extracts. The extracts contained two virus-related polypeptides that have the same apparent molecular weights as the two processed forms of virion-derived reverse transcriptase (p66 and p51). The formation of these two polypeptides depended on the co-expression of sequences located near the 5′ end of the *pol* gene, a region that is thought to encode a viral protease. This bacterial system appears to generate mature forms of the AIDS virus reverse transcriptase by a proteolytic pathway equivalent to that which occurs during virus infection of human cells.

THE STRUCTURE OF THE HUMAN immunodeficiency virus (HIV) genome (*1*) is similar to that of most retroviruses with respect to the placement of the *gag*, *pol*, and *env* genes (Fig. 1A). The HIV genome encodes several additional genes including *tat* and *art/trs* (*2*) as well as two open reading frames, A and B, which encode proteins of unknown function. By analogy to other retroviruses, expression of the HIV *pol* gene probably occurs by the occasional suppression of the *gag* translation termination codon thereby fusing the *pol* reading frame with the upstream *gag* reading frame (*3*).

The mammalian retrovirus *pol* gene typically encodes three protein products that express a total of four catalytic activities (*4*): a protease that cleaves the viral *gag* and *gag/pol* precursor polyproteins; the viral DNA polymerase [reverse transcriptase (RT)] with an associated ribonuclease H activity; and an integrase/endonuclease that functions in the integration of viral DNA into the host cell genome. The HIV *pol* gene is atypical with respect to other mammalian retrovirus *pol* genes in that it appears to encode four polypeptide products. Three of these *pol*-encoded polypeptides have been identified in HIV virions: two closely related forms of RT, p66 and p51, that differ in size (66 kD and 51 kD) but share a common amino terminus (*5, 6*); and a 34-kD protein (p34) that is the presumed integrase/endonuclease (*6*). The amino terminal sequences of these three polypeptides have been determined, thus permitting their assignment to specific regions of the *pol* gene open reading frame (*5, 6*). Like other mammalian retroviruses, the RT domain is encoded in the central portion, and the integrase/endonuclease is encoded in the 3′ portion of the *pol* gene open reading frame (Fig. 1B). Sequence comparisons (*1*) show that the remaining 5′ portion of the HIV *pol* gene encodes a polypeptide that shares homology with the protease domain of other avian and mammalian retroviruses.

Two plasmids were constructed containing HIV *pol* gene sequences under the control of the inducible *lac* promoter. The plasmids differ in the presence or absence of the presumed protease domain (Fig. 1C). The first plasmid, pBRT1prt$^+$, contains the protease and RT coding domains, starting with a Bgl II site located at the fifth codon of the *pol* open reading frame and terminating at an Eco RI site located in the middle of the integrase coding domain. The second plasmid, pBRT3prt$^-$, contains only the RT domain, starting with a Hinc II site located nine codons upstream of the position corresponding to the amino terminus of p66/p51 (*5, 6*) and terminating at the Eco RI site in the middle of the integrase coding domain.

Extracts were prepared from uninduced and induced bacterial cultures containing either the parental plasmid without a viral insert (pIBI20) or one of the pBRT plasmids. The presence of RT activity was detected by assaying bacterial cell extracts for RNA-dependent DNA polymerase activity with a homopolymeric polyribonucleotide template with a complementary oligodeoxy-

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