(rat) into mouse fibroblast cells after ligation with the HSV1TK gene, show a pattern of gene amplification similar to that observed in the parent strains from which these DNA sequences were isolated. The region of the rPRL gene 5′ end flanking sequences designated 17-1 (Fig. 1) is not amplified in response to BrdUrd in either of the GH cell strains or in transfectants derived with hybrid DNA containing these sequences (Fig. 2B).

The 3.8-kb 5′ end PRL gene–flanking region complementary to 17-2 (Figs. 1 and 3A) and PRL structural gene regions ($cDNA_{PRL}$ hybridizable sequences) (Fig. 3B) are likewise amplified only in BrdUrd-treated transfectants derived with hybrid DNA containing the HSV1TK and 5′ end DNA segment from PRL^- BrdUrd-responsive GH cells (Fig. 3, transfectants 11 and 26). Transfer of the hybrid DNA, HSV1TK-$rPRL^+$ BrdUrd-nonresponsive cells does not affect the levels of these sequences in BrdUrd-treated transfectants (Fig. 3, A and B; transfectants 55 and 66). These results suggest that the basic difference between the PRL gene of PRL^- and PRL^+ GH cells, in relation to BrdUrd-induced PRL gene amplification, is linked to this region (10.3-kb fragment) of the cellular DNA.

A 20-kb length of DNA at the 3′ end of 17-1 is amplified in BrdUrd-responsive GH cells. Cloned 10.3-kb DNA sequences of this region—the sequences complementary to 17-1 and 17-2 of the BrdUrd-responsive GH cells—when transferred to mouse fibroblast cells, showed a similar pattern of gene amplification in the transfectants. The HSV1TK gene, which is ligated at the 5′ end of the 10.3-kb rat DNA sequence, is also amplified in the transfectants. This suggests that the HSV1TK gene is located in the transfectant close to the 10.3-kb DNA segment of BrdUrd-responsive cells. As the 10.3-kb DNA segment has a Bam HI site at the 5′ end and an Eco RI at the 3′ end, the HSV1TK gene with Bam HI sites at both ends would be expected to anchor only at the 5′ end of the 10.3-kb DNA segment in the transfectants. Thus it may be postulated that the hybrid DNA retains its organization in the transfectants. However, this has yet to be established. Our results show that information responsible for the BrdUrd-induced gene amplification, which we have termed "amplicon": (i) is located within the 10.3-kb 5′ end flanking region of the PRL gene of BrdUrd-responsive GH cells, (ii) can be transferred from one cell type to the other, and (iii) induces amplification of another gene placed adjacent to it. The results also suggest that the structure of the amplicon region in the 10.3-kb segment at the 5′ end of the PRL gene differs in cells responsive and nonresponsive to BrdUrd.

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Distinctive Termini Characterize Two Families of Human Endogenous Retroviral Sequences

Abstract. *Human DNA contains many copies of endogenous retroviral sequences. Characterization of molecular clones of these structures reveals the existence of two related families. One family consists of full-length (8.8 kilobases) proviral structures, with typical long terminal repeats (LTR's). The other family consists of structures which contain only 4.1 kilobases of* gag-pol *sequences, bounded by a tandem array of imperfect repeats 72 to 76 base pairs in length. Typical LTR sequences that exist as solitary elements in the genome were cloned and characterized.*

Like the DNA's of other vertebrates (*1*), human DNA contains multiple germline copies of retroviral sequences. These endogenous structures were first identified and selected from a genomic DNA library through the use of two cross-species, low-stringency DNA hybridizations: the first, using a subclone of a murine leukemia virus to screen an African green monkey genomic library (*2*), and the second, using a subclone of the African green monkey retroviral sequence to probe a human genomic library (*3*). The first clone obtained, which displays considerable deduced amino acid homology to murine retroviruses in *gag* and *pol*, was used to screen the human library (*4*) under high-stringency conditions to obtain highly related clones (*5–7*). Since retroviral sequences are bounded by long terminal repeats (LTR's), we sought to localize such structures at the 5′ and 3′ termini of the cloned retroviral sequences. We now describe the nucleotide sequence of two distinct types of termini that were found.

The location of cross-hybridizing termini within the human endogenous retroviral clones was determined by Southern blotting (*8*) (data not shown). One type of repeat discovered in clone 4-1 (Fig. 1A) resembled a typical LTR and defined a structure with the size expected for proviral DNA [8.8 kilobases (kb)] (*6*). Additional clones with typical LTR's were obtained by screening the human genomic library with a 1.1-kb restriction fragment containing the 3′ LTR of clone 4-1 (Fig. 1A, box a). Of numerous positive plaques picked, 82 percent reacted with only the 3′ flanking cellular DNA component of the probe. The other 18 percent were equally divided between clones of the full length type, including clone 4-14 (Fig. 1A), and solitary LTR clones, which reacted only with a specific LTR probe (*6*), but not with *gag*, *pol*, or *env* probes.

The second type of repeating element, present in clone 51-1 as well as other clones having a similar restriction map (*5*), defined a truncated 6-kb retroviral segment (Fig. 1B).

The full-length and truncated retroviral sequences (Fig. 1, A and B, respectively) share a common 4.1-kb stretch of *gag-pol* sequence (*5, 9*) (Fig. 1B). Highly conserved restriction sites that reflect this sequence similarity are shown in Fig. 1: a Hind III site at 2.8 kb, Eco RI

sites at 3.9 and 4.9 kb, and a Bam HI site at 3.95 kb.

The sequence of the terminal repeating elements associated with the full-length retroviral structures in clones 4-1 and 4-14 (Fig. 2, A and B) exhibits features characteristic of LTR's (*10*) including a putative TATA box (T, thymine; A, adenine), polyadenylation signal, and, at the ends, imperfect inverted repeats. A polypurine tract (*11*) precedes the 3′ LTR's and a putative transfer RNA (tRNA) primer binding site follows the 5′ LTR's. The latter sequence is not complementary to proline-tRNA, the usual mammalian type C primer (*12*), but it is an almost perfect match for a rat glutamic acid–tRNA (*13*). Both 5′ and 3′ LTR sequences from the same clone show more than 90 percent base matching, while only a 73 percent match is seen between the two clones, due to differences in the U3 region of the LTR's. While structurally similar to known LTR's, the sequence of these human LTR's does not resemble other known LTR sequences such as in the baboon endogenous virus (*14*) or a chimp-related human endogenous clone (*15*). Comparison with LTR sequences that are conserved among many mammalian type C LTR's (*16*) reveals only a short match with sequences which follow the polyadenylation signal and with a small region near the end of direct repeats in some mammalian LTR's. A 4-base pair (bp) direct repeat flanks the provirus in clone 4-14; this feature is absent from clone 4-1, probably a result of a deletion of 5′ flanking cellular DNA (see below).

The sequence of repeats associated with the second (truncated) class of retroviral sequences consists of tandem arrays of an imperfect repeating unit, approximately 72 to 76 bp in length.

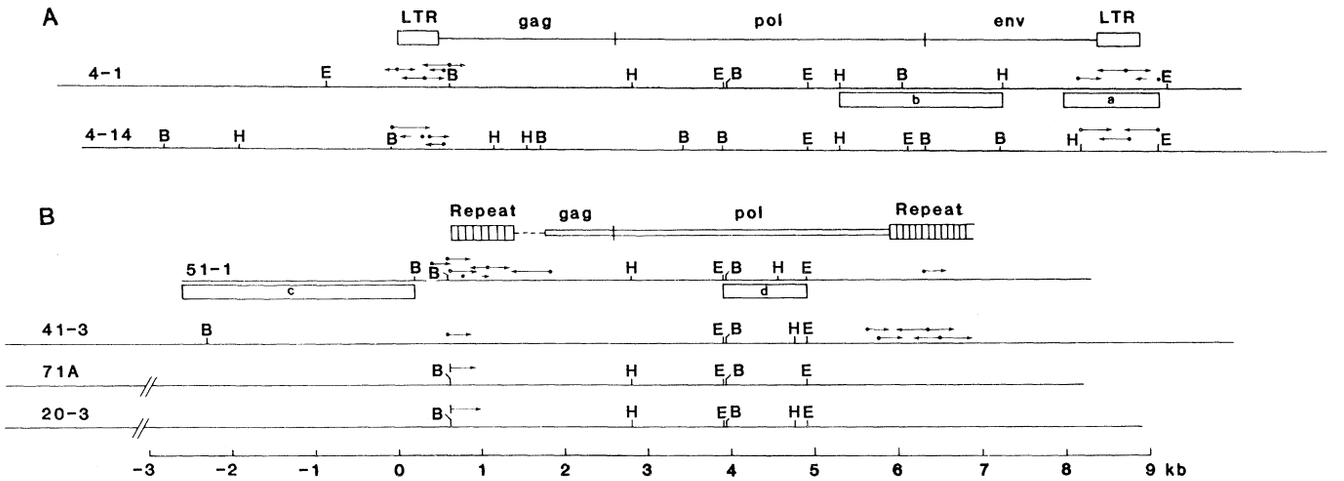

Fig. 1. Clone structure and sequence strategy. (A) Two DNA clones with full-length retroviral structures are aligned with a typical provirus schematic. (B) Four DNA clones with truncated retroviral structures are aligned with a schematic. The scored boxes indicate the repeat units, the open bar the 4.1 kb of *gag-pol* sequence shared with the full-length family, and the dotted line a nonretroviral AT-rich (adenine, thymine) sequence. The scored box on the 3′ side is open, as the 3′ boundary of that repeat has not been determined. A and B are drawn in alignment with each other. E, Eco RI; B, Bam HI; and H, Hind III. One additional unmapped Bam HI site exists in 4-14 between positions 6.3 to 7.2. Boxes a to d indicate restriction fragments that were used as probes (see text). Arrows denote the length and direction of sequencing. Dots indicate kinase sites for Maxam-Gilbert sequencing (*28*). Vertical lines at the beginning of arrows denote cloning sites for M13-Sanger sequencing (*29*).

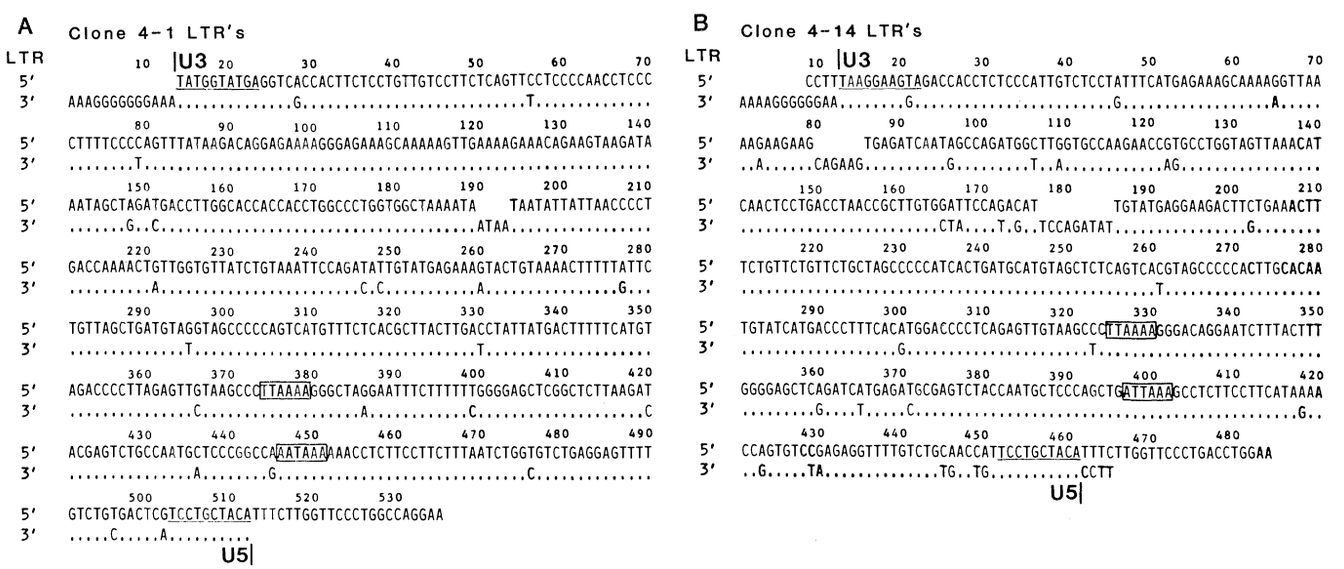

Fig. 2. Nucleotide sequence of LTR's. Each sequence begins with the polypurine tract which precedes the 3′ LTR and ends with an 18-bp putative tRNA binding site just beyond the 5′ LTR. Inverted repeats are underlined. Putative TATA and polyadenylated signal sequences are boxed. The 3′ LTR sequence is shown only when it differs from the 5′ LTR. Blank spaces in the 5′ LTR sequences indicate deletions relative to the 3′ LTR's. A 4-bp direct repeat is shown flanking the 4-14 LTR's.

ment has occurred at the precise 5′ viral-cell junction of clone 4-1, suggesting that LTR's may be involved in genomic DNA alterations.

The representation of both families of retroviral sequences in the genome has been evaluated by Southern blotting. The use of Kpn I (a "no-cut" or "one-cut" enzyme for human endogenous retroviral sequences) on five different human DNA's (Fig. 5) permits three major inferences. First, there are many copies of these sequences in human DNA, as the number of bands should approximate the copy number in this experiment. When probes which hybridize to both families are used, 37 Kpn I bands can be seen (Fig. 5) with the *pol-env* probe (Fig. 1A, box b) or with a *pol* probe (Fig. 1B, box d) (not shown). Since some of the bands are intense (presumably recruited), the copy number is much higher than 37. The use of probes specific for the full-length and truncated family yields 27 and 23 Kpn I bands, respectively, including some intense bands in each (not shown). Hence, the two families are approximately equally represented in the genome, and the total copy number detected with our probes is likely to be as many as 35 to 50 copies for each family, as judged from quantitative dot blot hybridization experiments (not shown). The second inference is that acquisition of new endogenous retroviruses or movement of existing ones in the germ line must occur rarely or not at all in humans, as there is virtually no polymorphism among five different individuals. Third, since the use of Kpn I detects either fragments that extend from 5′ flanking DNA to 3′ flanking DNA, or viral-cell junction fragments, the existence of recruited bands implies conservation of flanking DNA restriction sites among some endogenous retroviral sequences. This result has been reported for cloned feline leukemia virus-related endogenous sequences (*21*) and we have confirmed it with cloned human DNA (*22*). As with the truncated family, a process of DNA amplification involving both retroviral and flanking sequences is an attractive hypothesis. This type of amplification may have involved a progenitor of the endogenous retroviral sequence in clone 4-1. Subsequent excision of one of the amplified copies would explain the flanking DNA conservation between 4-1 and the target duplication clone (Fig. 4).

As cellular genes, endogenous retroviruses have been subjected to modifications, perhaps with adaptive value, over evolutionary time spans. Structural modifications affecting human endogenous

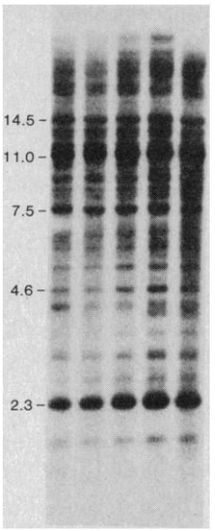

Fig. 5. Genomic blot hybridizations, comparing five individuals. Five human DNA's were treated with Kpn I and hybridized to a *pol-env* probe (Fig. 1A, box b). The probe lies 3′ to known Kpn I sites within the retroviral portion of our clones. Band sizes are in kilobases.

retroviral sequences include internal and 5′ terminal deletions which we have observed among some of our full-length-type clones (*23*) as well as the process which gave rise to the truncated class of clones described above. The complete or partial excision of retroviral sequences is another potentially adaptive change. The retention of a solitary LTR following a partial retroviral excision might influence the transcription of nearby cellular DNA (*17*), but any such use of retroviral sequences for nonviral, cellular functions remains unproved.

Other adaptations might include alterations in availability of required tRNA primers, which in turn could affect infectivity. Unavailability of primer could also affect transposition events (if they occur) in which retroviral RNA is reverse-transcribed and reintegrated into the genome. Novel, nonproline primer binding site (PBS) sequences as we have seen in the human retroviral clones may prove to be common among endogenous retroviruses, as a similar finding in mice has been described (*24*). Perhaps mammalian germlines were infected with more than one category of type C retroviruses, each with different tRNA PBS's. If so, the success (in terms of continued infectivity) of proline-tRNA PBS-containing type C viruses in mammals might be explained by the continued availability of proline tRNA, while the failure of other endogenous retroviruses to remain infectious might be due in part to alterations of structure or activity of the required cellular tRNA gene.

The elements we describe may represent only a fraction of those in human DNA's. Other investigators (*15, 25*) have also used cross-species, low-stringency hybridizations to detect and clone human endogenous type C–related retroviral elements, which may differ from those we describe. Among the endogenous retroviral elements we have examined are some that are spontaneously expressed as messenger RNA in human tissue; although there is no conclusive evidence for either the expression of the truncated family elements or for an enhancer function of their 72- to 76-bp terminal subrepeating units (*26*), our finding of RNA transcripts in various human tissues which anneal to the LTR segment of clone 4-1 implies that some human LTR's are actively functioning as enhancers and promotors (*6*). Such functional LTR's, if transposed or translocated into new locations, might play a role in malignant transformation or might act as mutagens to produce genetic disease.

Note added in proof: It was recently pointed out to us (*27*) that the cellular sequences flanking the 3′ LTR of clone 4-1 as well as the sequences associated with the putative target duplication (Fig. 4) are part of the Kpn I family of repeated human DNA.

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Most of the sequence data was obtained for the 5′ terminal repeat structure of clone 51-1 and the 3′ terminal repeat structure of clone 41-3 (Fig. 1B).

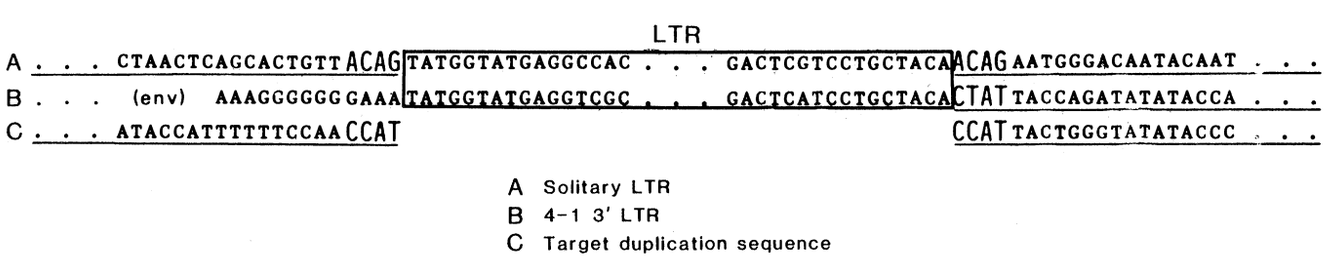
Eight tandem subrepeating units are present within the 5′ repeat structure of clone 51-1. At least 13 subrepeating units exist within the 3′ repeat structure of clone 41-3. Nucleotide sequence and length variation occur among the subrepeats, both within a single repeat structure (Fig. 3A) and between 5′ and 3′ repeat structures (Fig. 3B). When 5′ repeat structures among different clones or 3′ repeat structures among different clones are compared, the variation is considerably less. In fact, the homology is great enough (more than 90 percent) that the repeat structures in two clones can be precisely aligned with each other despite their content of similar subrepeating units (Fig. 3C), an indication of a possible drift of nucleotide sequence (Fig. 3) among the terminal repeat structures of a truncated retroviral element, with subsequent DNA amplification. The result is that many truncated elements now exist in which the 5′ repeat structures resemble each other more than they resemble their 3′ counterparts, and vice versa. The amplification unit must have encompassed more than the retroviral and terminal repeat sequences, since we have been able to select clones containing exclusively these truncated retroviral elements from a genomic DNA library with the use of a 5′ flanking DNA probe from one of the truncated family clones (Fig. 1B, box c) (*5*).

These truncated retroviral elements may have originated from the deletion of a full-length provirus, accompanied by the proliferation of the flanking subrepeating units, perhaps through a series of tandem duplications. They are unlikely to be a cellular progenitor of viral *gag-pol* genes because, as sequence analysis reveals, the retroviral-related portion begins 560 bp after the beginning of *gag* and ends 540 bp before the end of *pol* (*9*).

One of the clones containing a solitary LTR was analyzed by nucleotide sequencing. The LTR is identical in length to and exhibits 95 percent base matching with the LTR's of clone 4-1. A 4-bp direct repeat immediately flanks the solitary LTR (Fig. 4). This structure most likely represents a partial excision of a full-length provirus through homologous recombination between the LTR's (*17*) but could reflect the transposition of an LTR element. Type C–related solitary LTR's have been described in other species, including chickens (*17*), cats (*18*), and mice (*19*).

We also sequenced one of the many clones which were obtained from the library with the 1.1-kb probe located at the 3′ end of clone 4-1 (Fig. 1A, box a), but which annealed only to the 3′ flanking cellular DNA component of that probe and not to any retroviral sequences. This clone contained a stretch of DNA that exhibited a collinear 75 percent base match with the 3′ flanking DNA of 4-1, extending exactly to the integration site in 4-1, where there was a 4-bp direct repeat (Fig. 4). If this finding represents a proviral excision, leaving behind a direct repeat, it resembles the excision of a *Ds* element in maize in which the direct repeat, slightly modified, was retained (*20*). As mentioned above, the 4-1 retroviral element does not contain a 4-bp direct repeat that immediately flanks the proviral DNA of other human clones. In fact, the nucleotide sequence 5′ to the solitary target duplication (49 bp determined) did not match the 5′ flanking cellular DNA of 4-1 (125 bp determined), suggesting that the latter sequences have been deleted or rearranged. This deletion or rearrange-

Fig. 3 (left). Nucleotide sequence of subrepeats from the truncated retroviral family. (A) Thirteen contiguous subrepeats from the 3′ repeat of clone 41-3 are shown in "best-fit" alignment. The identity of two nucleotides, labeled N, was not determined. (B) A 5′ and a 3′ subrepeat are aligned to show maximum base matching as noted by asterisks. (C) Diagram indicates that 5′ or 3′ terminal repeat structures can be precisely aligned despite their content of subrepeating units. There is more than 90 percent homology between a and a′, b and b′, x and x′, while other combinations (such as a and b, a and b′, and a and x) are less than 70 percent homologous, as seen in (B). Fig. 4 (below). Target duplication sequences. The sequence of the LTR-cellular DNA junctions of the (A) solitary LTR and the sequence in the related flanking DNA clone at the target duplication site are aligned with the (B) 3′ LTR and adjacent sequences from the full-length-type clone 4-1; (C) is the target duplication sequence. The LTR sequences are boxed. Nonretroviral (cellular) sequences are underlined. Large letters indicate the 4-bp direct repeat which flanks the solitary LTR, the 4 bp which lie 3′ to the 3′ LTR of clone 4-1 and the 4-bp target duplication in the related flanking DNA clone.



A Solitary LTR
B 4-1 3′ LTR
C Target duplication sequence

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30. We thank Roy Repaske and Raymond O'Neill for introducing Maxam-Gilbert sequencing; Arifa Khan for introducing M13-Sanger sequencing; Karen Weck for mapping solitary LTR subclones; M. David Hoggan and Johnna Sears for the blot hybridization in Fig. 5; Charles Buckler for assistance in data analysis; and Willard Davis and J. Harry Belcher for technical assistance.

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Alzheimer's Disease Brain: Alterations in RNA Levels and in a Ribonuclease-Inhibitor Complex

Abstract. *A macromolecular alteration occurs at the posttranscriptional level in the Alzheimer's disease (AD) brain. Compared with age-matched controls, total cellular RNA and polyadenylated RNA were substantially reduced in the AD cortex with many neuritic plaques and neurofibrillary tangles. RNA changes are associated with a significant increase in alkaline ribonuclease activity due to an abnormality in the ribonuclease-inhibitor complex. The decrease in protein synthesis in the AD brain, previously observed in patients severely affected with AD, and in translation systems in vitro with AD cortical messenger RNA, may be partly related to an enzyme-inhibitor alteration that affects RNA levels and activity. Decreased protein synthesis therefore may contribute to the characteristic decline in certain neurotransmitter enzymes and to the loss of neurons in the AD brain.*

The Alzheimer's disease (AD) brain is characterized by loss of specific neurons, and the degree of dementia can be correlated with cerebral neuronal degeneration involving neuritic (senile) plaques and neurofibrillary tangles and a decline in choline acetyltransferase activity (*1*). Although it is unlikely that the dramatic intra- and extracellular pathology occurs by processes unrelated to transcriptional or translational control mechanisms (or both), data concerning macromolecular alterations that may be relevant to the etiology of AD are limited. Our initial studies showing a decline in protein synthesis directed by AD brain messenger RNA (mRNA) in vitro (*2*) were independently confirmed by positron emission tomography studies that showed decreased incorporation of methionine into brain protein of patients afflicted with AD (*3*). Various factors may contribute to the decline in protein synthesis in affected regions of the AD brain. We have examined one of these factors, namely that the severely affected AD cortex has decreased levels of ribosomal RNA (rRNA) and mRNA due to a posttranscriptional alteration at the level of RNA degradation (*4*).

Brain and other mammalian tissues contain a major "alkaline" ribonuclease, which is active at *p*H 7.2 to 8.0 (*5–7*). Unlike acidic ribonuclease, which is localized in lysosomes along with other acid hydrolases (*8*), the alkaline enzyme in brain homogenates is primarily localized in the soluble fraction, and to a lesser extent in nuclei and microsomes, and small amounts can be detected in other subcellular fractions (*9*); higher activities are present in neuronal rather than glial nuclei (*10*). The activity of alkaline ribonuclease is restricted by the presence of a bound inhibitor (*6*), a 50- to 60-kD protein (*11*), that is present in higher concentrations in brain than other organs (*6, 12*). Inhibitor from brain and liver supernatants has been used to stabilize polysome preparations (*13*). Because of the presence of inhibitor in tissue homogenates, the ribonuclease activity is relatively low and is referred to as free ribonuclease. When the inhibitor is inactivated by sulfhydryl-blocking reagents, such as *p*-chloromecuribenzoate (PCMB) (*6*), the activity increases and is referred to as total alkaline ribonuclease.

Control samples of cerebral cortex were obtained at autopsy from four individuals that had died with no history of dementia or other neuropsychiatric disease; the mean age at death was 72 ± 5.6 years and the postmortem interval was 8.7 ± 1.8 hours. On the basis of the clinical history of dementia and the histopathologic identification of both neuritic plaques and neurofibrillary tangles, the diagnosis of AD was established in six patients with a mean age at death of 70.8 ± 2.9 years; the interval between death and autopsy was 11.9 ± 2.9 hours [(*14*) and the legend to Fig. 1].

Total cellular RNA, consisting predominantly of ribosomal RNA, was prepared from the foregoing cortical specimens that had been preserved at −70°C and not thawed prior to extraction (*15*). The average yield from the control brain specimens was 100 ± 7.7 μg of RNA per gram of tissue as compared to 54.7 ± 9.5 μg/g from AD specimens (Fig. 1). The difference is statistically significant [$t(10) = 3.69$; $P < 0.01$] and represents a 45.3 percent reduction in the yield of RNA extracted from brain tissue containing both plaques and tangles as compared with controls. The relative decrease in AD RNA is consistent with measurements obtained by histologic staining of postmortem brain (*16*).

The foregoing total cellular RNA samples were used to prepare poly(A)$^+$ (polyadenylated) RNA by oligo(dT)-cellulose (deoxythymidylate) column chromatography (*17*). These described procedures applied to both control and AD postmortem brains allow the preparation of poly(A)$^+$ RNA that retains mRNA activity during in vitro protein synthesis (*2, 15*). The average yield of poly(A)$^+$ RNA from control brains was 2.8 ± 0.4 μg/g; the average yield of poly(A)$^+$ RNA from the series of AD specimens was 1.0 ± 0.2 μg/g, a significant relative reduction [64.3 percent decrease, $t(10) = 3.85$, $P < 0.01$] compared with controls. When data from the foregoing brains were analyzed together with data from a larger series of control and AD specimens a significant correlation could not be established between the yield of total cellular RNA [$r = -0.32$; $t(14) = 1.16$; $P > 0.2$] or of poly(A)$^+$ RNA [$r = -0.47$; $t(14) = 1.96$; $P > 0.05$] and the postmortem interval in the range of 3.5 to 25 hours.

Cortical specimens adjacent to those taken for RNA preparations were used to measure the free and total alkaline ribonuclease activity at *p*H 7.5 (Fig. 2); like acidic ribonuclease, alkaline ribonuclease retains its activity in previously frozen tissue (*20*). Denatured mammalian [^{3}H]rRNA was added to serve as a substrate. The mean free alkaline ribonuclease activity (per milligram of tissue) was 39.3 ± 6.2 units in control specimens (Fig. 2). Among AD brains the mean free ribonuclease activity was 73.6 ± 10.5 units, which represented a significant increase (87.3 percent) over control values [$t(10) = 2.80$; $P < 0.05$].

In the presence of PCMB (1 m*M*) all control cortical samples showed a substantial increase (133.1 percent) in alkaline ribonuclease which ranged from 35 to 62 units with a mean increase of 52.3 ± 6.2 units (Fig. 2B). By contrast, the mean increase in alkaline ribonucle-