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Tissue-Specific Expression of Kallikrein-Related Genes in the Rat†

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ABSTRACT: Four distinct kallikrein-related mRNAs (PS, S1, S2, and S3), encoded by members of a multigene family, are selectively expressed in various combinations in several rat tissues. Although closely related along most of the mRNA sequence, the four mRNAs can be selectively detected with synthetic oligonucleotide probes complementary to highly variable mRNA subregions. PS mRNA, which encodes an enzyme with true kallikrein activity, is present at high levels in the submaxillary gland, pancreas, and kidney. S1 mRNA, which encodes an enzyme similar to the PS kallikrein, is detected only in the submaxillary gland and is present at one-fifth the PS mRNA level. S2 mRNA, which encodes the enzyme tonin, is present in the submaxillary gland at half the PS mRNA level and at a slightly higher level in the prostate. S3 mRNA, which encodes an enzyme very similar to tonin, is present in the submaxillary gland at one-tenth the PS mRNA level and in the prostate at about the same level as tonin mRNA.

The kallikrein-like enzymes are homologous serine proteases encoded by a multigene family. True kallikreins (EC 3.4.21.8) selectively cleave the protein kininogen, found in the blood and in the interstitium of most tissues, to release the vasodilatory peptides bradykinin or lysylbradykinin [reviewed by Fiedler (1979)]. Other members of the kallikrein-like enzyme family cleave other prohormones: tonin cleaves angiotensinogen (Boucher et al., 1974), the γ subunit of nerve growth factor processes the precursor of nerve growth factor (Thomas et al., 1981), and the epidermal growth factor binding protein processes the precursor of epidermal growth factor (Frey et al., 1979). The kallikrein-like enzymes most likely function within the organ in which they are synthesized, and their presence

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may reflect the particular needs of peptide hormone maturation within a specific cell type or tissue.

In the rat and the mouse, the submaxillary gland contains the highest levels of kallikrein-like enzymes (Frey et al., 1968; Brandtzaeg et al., 1976). Kallikrein and kallikrein-like enzymes are also found in a number of other exocrine tissues, such as the sublingual and parotid glands (Brandtzaeg et al., 1976), the kidney (Orstavik et al., 1976), the pancreas (Orstavik & Glenner, 1978), the lacrimal glands, the sweat glands, the guinea pig coagulating gland, and the intestinal mucosal glands [for a review, see Nustad et al. (1978)]. Recently, mRNA related to pancreatic kallikrein was detected in several rat tissues (Swift et al., 1982), including the submaxillary gland, which has the highest level, the pancreas, kidney, and parotid gland, as well as the spleen, in which kallikrein had not been previously reported. These results establish that kallikrein-like enzymes are actually synthesized in these tissues.

In the preceding report (Ashley & MacDonald, 1985), we described the analysis of four rat submaxillary gland mRNAs encoding kallikrein-like enzymes. The PS-type mRNA is a prominent submaxillary gland mRNA also found in the pancreas (Swift et al., 1982) and kidney. It encodes a serine protease with true kallikrein activity. S1 mRNA encodes an enzyme very similar to tissue kallikrein, but of unknown function. S2 mRNA encodes the serine protease tonin, which can process angiotensinogen to angiotensin II in vitro (Boucher et al., 1974). S3 mRNA encodes a tonin-like enzyme of unknown cleavage specificity and function. The present study describes the differential expression of the genes encoding these four kallikrein-like enzymes in the rat. Using synthetic oligodeoxynucleotide probes for each of the four kallikrein-related mRNAs, we have characterized the tissue-specific expression patterns of these kallikrein-related mRNAs and have identified the cloned genes that encode rat kallikrein and tonin.

Experimental Procedures

Preparation of Rat Tissue RNAs. RNA was isolated from submaxillary gland, pancreas, prostate gland, kidney, spleen, parotid gland, testes, and liver of male Sprague-Dawley rats by using a slightly modified version of the guanidine thiocyanate extraction procedure (Ashley & MacDonald, 1985; Chirgwin et al., 1979). The total RNA of each tissue from 10 rats was pooled to isolate polyadenylated RNA by oligo(dT)–cellulose chromatography (Aviv & Leder, 1972; MacDonald et al., 1982).

Northern Blot Analysis of Glandular Kallikrein mRNAs. Polyadenylated RNAs were resolved in 2% agarose gels containing methylmercury hydroxide (MacDonald et al., 1982). The RNA was transferred to GeneScreen (New England Nuclear) hybridization membrane by electroblotting (Stellwag & Dahlberg, 1980). After being baked at 80 °C in a vacuum oven for 2 h, the GeneScreen filter was hybridized to a ^{32}P-labeled single-stranded cDNA probe as described previously (Ashley & MacDonald, 1984).

DNA-Excess Filter Hybridization To Determine Kallikrein-Related mRNA Concentrations in Rat Tissues. Samples containing 10 μg of the rat pancreatic kallikrein cDNA clone pcXP39 (Swift et al., 1982) were bound to 11-mm diazobenzyloxymethyl (DBM) filter circles (Schleicher & Schuell) as described by Stark and Williams (1979). The cDNA insert of pcXP39 encodes the 3′ end of the PS-type pancreatic kallikrein mRNA, from codon 70 to the poly(A) tract (Swift et al., 1982). Polyadenylated RNA from the eight tissues mentioned above was hydrolyzed in 0.1 N NaOH at room temperature for 15 min, neutralized with HCl, and precipitated with ethanol. The base-hydrolyzed RNAs were then end labeled with [γ-^{32}P]ATP and polynucleotide kinase (P-L Biochemicals) (Maxam & Gilbert, 1980). The unincorporated nucleotide was removed by centrifugation through a Bio-Gel P30 column (MacDonald et al., 1982), and ^{32}P incorporation was determined by a DE81 filter assay (Maniatis et al., 1982). Approximately 5×10^5 cpm of ^{32}P-labeled RNA were hybridized to the filter-bound plasmid DNA in 100 μL of 50% formamide hybridization buffer (Ashley & MacDonald, 1984) overnight at 42 °C. The filters were then washed, and the hybridized RNA was eluted with NaOH as described (Stark & Williams, 1979). The [^{32}P]RNA bound to each filter was determined by scintillation counting in NCS tissue solubilizer (Amersham) and Omnifluor (NEN). Background hybridization was determined by hybridizing filter-bound pBR322 with ^{32}P-labeled liver RNA.

Determination of Heteroduplex Dissociation Temperatures. Samples containing 5 μg of polyadenylated RNA from the eight rat tissues indicated above were bound to 11-mm DBM filter circles as described (Stark & Williams, 1979). Individual filters were then prehybridized for 2 h at 42 °C in 50% formamide hybridization buffer and incubated overnight at 42 °C in 100 μL of 50% hybridization buffer containing 5×10^5 cpm of a single-stranded rat pancreatic kallikrein cDNA probe prepared from an M13mp8 subclone of the cDNA insert of pcXP39 (Ashley & MacDonald, 1984). The filters were washed and the bound hybrids melted stepwise in 0.3 M NaCl, 0.03 M sodium citrate, and 50% formamide buffer according to Kafatos et al. (1979). The stepwise release of ^{32}P-labeled cDNA was determined by Cerenkov counting the filters.

Northern Blot Analysis with Synthetic Oligonucleotide Probes. Polyadenylated RNAs were resolved in agarose gels containing formaldehyde (Lehrach et al., 1977) and transferred to Zeta-Probe (Bio-Rad) hybridization membranes by electroblotting. The filters were baked for 2 h at 80 °C in vacuo, washed overnight in 6× SSC (SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.5% sodium dodecyl sulfate (SDS), and prehybridized overnight in 6× SSC hybridization buffer (6× SSC, 1 mM sodium pyrophosphate, 5× Denhardt's solution, and 100 μg/mL yeast tRNA) at a temperature 5 °C below the calculated T_d of the oligonucleotide probe to be used. T_d's were calculated from the empirical rules of Suggs et al. (1981): 4 °C for each G-C base pair and 2 °C for each A-T base pair. The synthetic oligonucleotide probes were synthesized by using phosphoramidite chemistry either manually with the New England Biolabs synthesis kit or mechanically with an Applied Biosystems 380A automated oligonucleotide synthesizer. These oligonucleotide probes were end labeled with [γ-^{32}P]ATP (NEN) and polynucleotide kinase (Maxam & Gilbert, 1980). Hybridization was carried out under the conditions used for prehybridization with the addition of 3×10^6 cpm of labeled oligonucleotide probe per milliliter of hybridization buffer. The filters were washed in three 500-mL changes, for 20 min each, of 6× SSC and 1 mM sodium pyrophosphate, with 0.5% SDS added to the first wash, at a temperature 10 °C below the T_d of the oligonucleotide probe used. After being air-dried, the filters were exposed to X-ray film at −80 °C with an intensifying screen.

Southern Blot Analysis. Rat liver DNA was digested to completion with *Bam*HI, *Eco*RI, or *Hin*dIII. Samples containing 10 μg of DNA were electrophoresed through 1% agarose gels and transferred to nitrocellulose filters according to Southern (1975). The filters were prehybridized overnight at 42 °C in 50% formamide hybridization buffer. Hybridization to the nick-translated (Rigby et al., 1977) insert of the pancreatic kallikrein cDNA clone pcXP39 (Swift et al., 1982)

was carried out under the same conditions. The filter was washed in three 500-mL changes of 0.1× SSC at 55 °C with 0.1% SDS added to the first wash, for 20 min each. After being air-dried, the filter was exposed to preflashed X-ray film at −80 °C with an intensifying screen.

Analysis of Kallikrein-Related Genes. A rat genomic DNA library was constructed (Maniatis et al., 1982) with the bacteriophage λ vector L47.1 (Loenen & Brammar, 1980) and rat liver DNA partially digested with *Sau*3A. A total of 24 recombinant λ clones bearing kallikrein-related gene sequences among a total of 10^6 screened were detected by hybridization (Ashley & MacDonald, 1984) with pcXP39 and pcXP130 (Swift et al., 1982), which represent the 3′ and 5′ ends of pancreatic kallikrein (PS-type) mRNA, respectively. To determine the identity of the cloned kallikrein-related genes, the cross-hybridizing recombinant λ phage DNAs were digested with *Hin*dIII, electrophoresed through 1% agarose gels, transferred to nitrocellulose filters according to the method of Southern (1975), and hybridized with the individual oligonucleotide probes under the conditions described above.

Gene Copy Number Quantification. Samples of a full-length kallikrein cDNA clone, pPS-J (Ashley & MacDonald, 1985), containing the mass equivalent of 0–25 copies of the represented kallikrein sequences as would be present in 1 μg of rat genomic DNA were denatured in 0.2 N NaOH, 10× SSC, and 30 μg/mL yeast tRNA at 80 °C for 15 min. The samples were neutralized by the addition of 2 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.5, to 0.25 M and spotted in triplicate onto nitrocellulose by using a Minifold microsample filtration manifold (Schleicher & Schuell). Sonicated rat liver DNA was denatured in the same manner, and 1-μg samples were applied to the nitrocellulose filter. After being baked in a vacuum oven at 80 °C for 2 h, the filter was hybridized with a single-stranded PS-type pancreatic cDNA probe prepared from an M13mp8 subclone of pcXP39 as described previously (Ashley & MacDonald, 1984). After autoradiography, the dots were cut out, and the bound radioactivity was determined by Cerenkov counting.

Quantification of mRNAs with Oligonucleotide Probes. Samples containing 1 μg of polyadenylated submaxillary gland RNA in 5× SSC were spotted onto nitrocellulose by using a Minifold apparatus. After the filters were baked, RNA dots (in triplicate) were hybridized with the individual four oligodeoxynucleotide probes in 6× SSC hybridization buffer (0.9 M NaCl). Preparations of ^{32}P-labeled oligonucleotides of similar specific radioactivity [(2.5–3) × 10^6 cpm/pmol] were used to ensure comparable hybridization results. The incubation temperatures used were as follows: 3′PSkal$_{21}$, 48 °C; 3′S1kal$_{16}$, 37 °C; 3′S2kal$_{21}$, 47 °C; 5′S3kal$_{21}$, 47 ° C. After the filters were washed and autoradiographed, the dots were cut out, and the radioactivity was measured by Cerenkov counting.

Results

Expression of Glandular Kallikrein mRNA in Rat Tissues. The presence in various rat tissues of RNA species with sequence homology to pancreatic kallikrein mRNA was demonstrated by Northern blot analysis of polyadenylated RNA using a single-stranded pancreatic kallikrein cDNA probe. The ^{32}P-labeled probe hybridized to single major bands in the RNA of submaxillary gland, pancreas, prostate gland, kidney, spleen, and parotid gland (Figure 1). A longer exposure of the filter revealed hybridization to RNA of testes and liver also. The principal hybridizing RNAs are approximately the same size (1.0 kilobase) but appear to be expressed at widely varying levels.

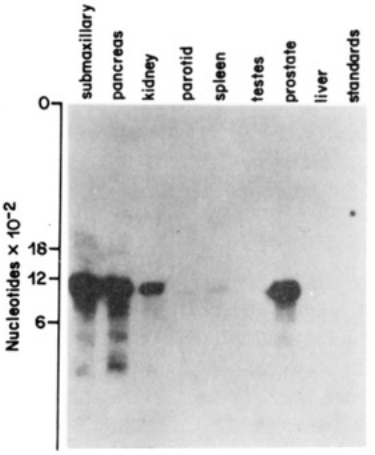

FIGURE 1: Tissue distribution of kallikrein-related mRNAs in the rat. Samples containing 1 μg (submaxillary gland) or 10 μg (other tissues) of polyadenylated RNA were electrophoresed through a methylmercury hydroxide–agarose gel, transferred to GeneScreen, and hybridized to ^{32}P-labeled pancreatic kallikrein cDNA. The hybridizing bands below the major bands at 1 kb represent degradation products in this autoradiogram overexposed to reveal hybridization in the parotid and spleen lanes. The lane marked standards contained *Hin*cII-digested φX174 DNA.

Table I: Level of Kallikrein-Related mRNAs in Various Rat Tissues

source of RNA	expt	cpm added	cpm hybridized	% hybridized	av % hybridized
submaxillary gland	1	5.0×10^5	2696	0.54	1.1
	2	4.6×10^4	1059	2.31	
	3	3.7×10^5	1980	0.54	
prostate gland	2	6.5×10^5	2543	0.39	0.37
	3	4.7×10^5	1624	0.34	
pancreas	1	5.0×10^5	416	0.08	0.18
	2	5.0×10^5	1770	0.35	
	3	5.2×10^5	580	0.11	
kidney	1	5.0×10^5	592	0.12	0.11
	2	1.5×10^5	209	0.14	
	3	4.1×10^5	264	0.06	
spleen	2	1.1×10^5	169	0.08	0.08
	3	3.7×10^5	263	0.07	
parotid gland	1	5.0×10^5	792	0.10	0.08
	2	1.7×10^5	167	0.08	
	3	3.9×10^5	233	0.06	
liver	1	5.0×10^5	148	0.03	0.03
	2	3.9×10^5	116	0.03	
	3	4.7×10^5	151	0.03	
testes	1	5.0×10^5	88	0.02	0.03
	3	5.2×10^5	187	0.03	

Kallikrein-related mRNA levels were quantified in each tissue by hybridization of ^{32}P-labeled polyadenylated RNA to a PS-type rat pancreatic kallikrein cDNA, pcXP39 (Swift et al., 1980), immobilized on DBM filter circles (Table I). The fraction of kallikrein-related sequences in the polyadenylated RNA of these tissues ranged from 0.03% (testes and liver) to 1.1% (submaxillary gland). These are approximate values, because mismatches between the pancreatic kallikrein cDNA probe and kallikrein-related RNAs lead to varying hybridization efficiencies.

To estimate the number and relatedness of the kallikrein-like mRNAs expressed in rat tissues, we measured the degree of sequence homology between pancreatic kallikrein mRNA and the kallikrein-related mRNAs in other tissues by measuring heteroduplex dissociation temperatures (T_d) (Figure 2). The complexity of the melting curves suggests that each tissue either contains more than one mRNA related to PS kallikrein mRNA or contains a single mRNA with domains that differ in their extent of sequence homology with the PS mRNA. The T_d of the submaxillary gland RNA–pancreas cDNA hybrids

Table II: Heteroduplex Dissociation Analysis of Rat Kallikrein-Related mRNAs

tissue	T_d (°C)	ΔT_d (°C)	% mismatch[a]
pancreas	59		
submaxillary gland	58.5	0.5	0.8
prostate gland	56.5	2.5	3.8
kidney	52.0	7.0	11
parotid gland	52.0	7.0	11
spleen	48.5	10.5	16
testes	48.5	10.5	16

[a] Calculated according to Jones et al. (1979); 1 °C $\Delta T_d \cong 1.5\%$ mismatch.

was almost identical with that of the homologous pancreas RNA–pancreas cDNA hybrids, indicating that the kallikrein-related mRNAs expressed in these tissues are very similar and likely include sequences that are identical. The lower thermal stability of the cDNA–mRNA heteroduplexes for the other tissues indicates that the kallikrein-related mRNAs differ from PS kallikrein mRNA by 3.8–16% (Table II). The five classes of T_d's for the different tissues suggest the presence of at least five different mRNAs of varying degrees of mismatch.

Expression of Four Distinct Submaxillary Gland Kallikrein-Related mRNAs in Other Tissues. Recently, four different, but closely related, kallikrein-like mRNAs expressed in the rat submaxillary gland have been characterized (Ashley & MacDonald, 1985). The nucleotide sequences of the four mRNAs are 85–94% homologous, and extended cDNA regions readily cross-hybridize. However, the nucleotide sequences within three short regions of the amino acid coding domains encompassing codons 18–27, 78–90, and 135–145 are variable (Ashley & MacDonald, 1985). Oligodeoxynucleotide probes complementary to the variable regions covering codons 136–142 (3′) or codons 78–84 (5′) were synthesized to distinguish the four mRNAs in hybridization experiments (Table III).

The patterns of expression of the four submaxillary gland kallikrein-related mRNAs in the various rat tissues were determined by Northern blot analyses. The oligomer complementary to the PS kallikrein mRNA (3′PSkal_{21}) hybridized to a 1.0-kilobase (kb) RNA species in submaxillary gland, pancreas, and kidney, in decreasing order of intensity (Figure 3a). The validity of the oligomer identification of PS mRNA has been confirmed by the identity of nucleotide sequences of the cloned cDNAs from pancreas and submaxillary gland (Ashley & MacDonald, 1985) and a partial cDNA from kidney (S. Clift, P. Ashley, and R. MacDonald, unpublished

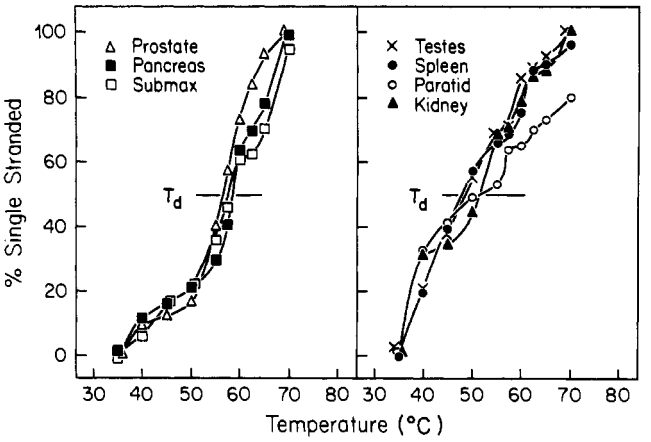

FIGURE 2: Melting profiles of tissue RNA–pancreas cDNA heteroduplexes. Filter-bound polyadenylated RNA from various rat tissues was hybridized with ^{32}P-labeled single-stranded pancreatic cDNA; the filters were washed, and the bound hybrids were melted as described under Experimental Procedures.

results). S1 mRNA was detected with the 3′S1kal_{16} probe only in submaxillary gland (Figure 3b). The S1 mRNA is slightly smaller (approximately 0.9 kb) than PS, S2, and S3 mRNAs. The remaining two kallikrein mRNAs, S2 and S3, are present in the submaxillary gland and in higher amounts in the prostate gland (Figure 3c,d).

To verify the specificity of hybridization of the oligonucleotide probes, the T_d of each oligonucleotide probe–mRNA heteroduplex was determined for all the tissue mRNAs which hybridized with the probes (Figure 4). The T_d's for each individual oligonucleotide were identical in each tissue where the hybridizing RNA was found. This suggested that each oligonucleotide probe hybridized to a single mRNA species. Under the melting conditions employed, single internal nucleotide mismatches would lower the observed T_d about 10 °C (Wallace et al., 1979); mismatches at ends have smaller, but still significant, effects on T_d values (Gillam et al., 1975).

The relative levels of the four kallikrein-related mRNAs in submaxillary gland RNA were determined by spotting polyadenylated RNA from rat submaxillary gland onto nitrocellulose and hybridizing separately to each oligonucleotide probe (Figure 5). Comparable hybridization kinetics for all four oligonucleotide probes were ensured by hybridization at 5 °C below the characteristic T_d determined for each oligonucleotide. The relative levels of PS kallikrein, S2/tonin, S1, and S3 mRNAs in the submaxillary gland are 1.0, 0.45, 0.22, and 0.09, respectively.

Table III: Oligonucleotide Probes Complementary to the Variable Regions of Kallikrein-Related mRNAs

	mRNA sequence[a]	oligomer designation	no. of differences with respect to S1kal	S2kal	S3kal
PS	mRNA...CATCACACCTGACGGATTGGA...	3′PSkal_{21}	10	11	12
S1	mRNA...CACCAACCCCTCTGAGTGGGA...	3′S1kal_{16}[b]		3	4
S2	mRNA...CACCAACCCCTCTGAGATGGT...	3′S2kal_{21}			3
S3	mRNA...GACCAACCCCTCTGAGATGAA...	ns[c]			
PS	mRNA...AACCAGGACCTCATATGGAAC...	ns	(≥0)	11	7
S1	mRNA...--------ACCTCATATGGAAC[d]...	ns		(≥7)	(≥4)
S2	mRNA...ATCCCACTCATCGTGACGAAT...	ns			5
S3	mRNA...ATCCCAGTCTTCATGAGGAAC...	5′S3kal_{21}			

[a] The first four mRNA sequences cover codons 136–142; the second four mRNA sequences cover codons 78–84. [b] This synthetic oligonucleotide is 16 nucleotides long, covers the S1 mRNA region underlined, and has the following number of mispairings with the other mRNAs: with PS, 10; with S2 and S3, 3. [c] None synthesized for this mRNA region; mRNA sequence shown for comparison only. [d] The truncated S1 cDNA clone ends with the sequence shown; the numbers in parentheses indicate the minimum number of differences with the other mRNAs.

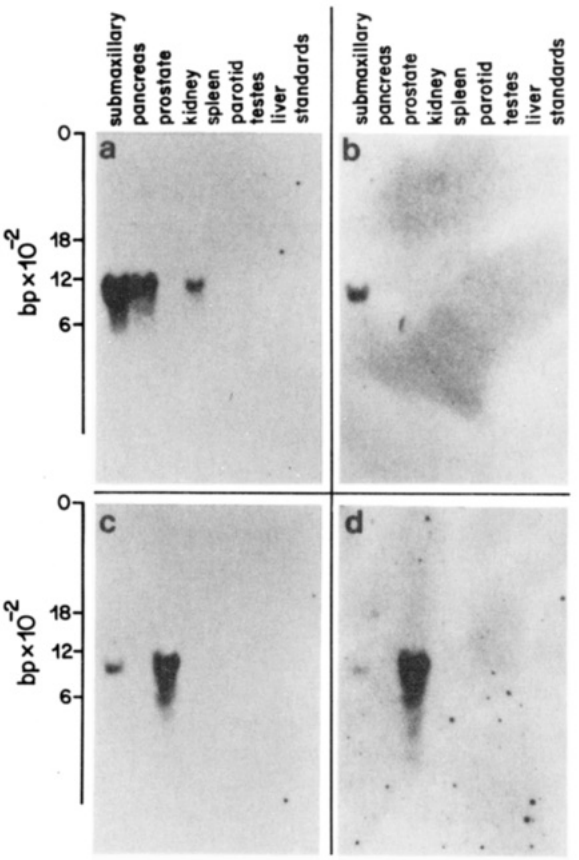

FIGURE 3: Tissue distribution of the four submaxillary gland kallikrein mRNAs in rat tissues. Samples contaiing 1 μg (submaxillary gland) or 5 μg (other tissues) of polyadenylated RNA were resolved by electrophoresis through a formaldehyde–agarose gel, transferred to Zeta-Probe, and hybridized to ^{32}P-labeled oligonucleotide probes specific for each kallikrein mRNA. The lane marked standards contained *Hin*cII-digested ϕX174 DNA. The oligonucleotide probes were (a) 3′PSkal$_{21}$, (b) 3′S1kal$_{16}$, (c) 3′S2kal$_{21}$, and (d) 5′S3kal$_{21}$.

Number of Kallikrein-Related Genes in the Rat Genome. Cloning four distinct kallikrein-related mRNAs indicated that at least four kallikrein-related genes are present in the rat genome (Ashley & MacDonald, 1985). Hybridization of the rat pancreatic kallikrein cDNA probe pcXP39 (Swift et al., 1982) to a Southern blot containing rat liver DNA digested with *Bam*HI, *Eco*RI, or *Hin*dIII revealed 7–10 cross-hybridizing gene fragments (Figure 6). These hybridization patterns suggested the existence of multiple genes coding for different members of the kallikrein family, although the number of cross-hybridizing genes could not be estimated accurately. Differences in the intensity of hybridization signals for certain restriction fragments may indicate the conservation of particular restriction endonuclease sites in several kallikrein genes that then yield hybridizing DNA fragments of similar size. Alternatively, divergence of the nucleotide sequence of some family members may limit the intensity of hybridization to some fragments.

A more accurate estimate of the number of kallikrein family genes was obtained with a quantitative dot blot assay of total rat genomic DNA. Samples of sonicated rat liver DNA were bound to nitrocellulose and hybridized with a PS cDNA hybridization probe. The extent of hybridization was measured by Cerenkov counting and compared to the extent of hybridization to standards containing 0–25 gene-equivalent copies of PS kallikrein cDNA per haploid genome. Rat DNA contains approximately eight gene copies which cross-hybridize with the kallikrein probe (Figure 7). Since divergent family members would be expected to hybridize with the PS gene probe less efficiently, the estimate of eight gene copies is likely a lower limit.

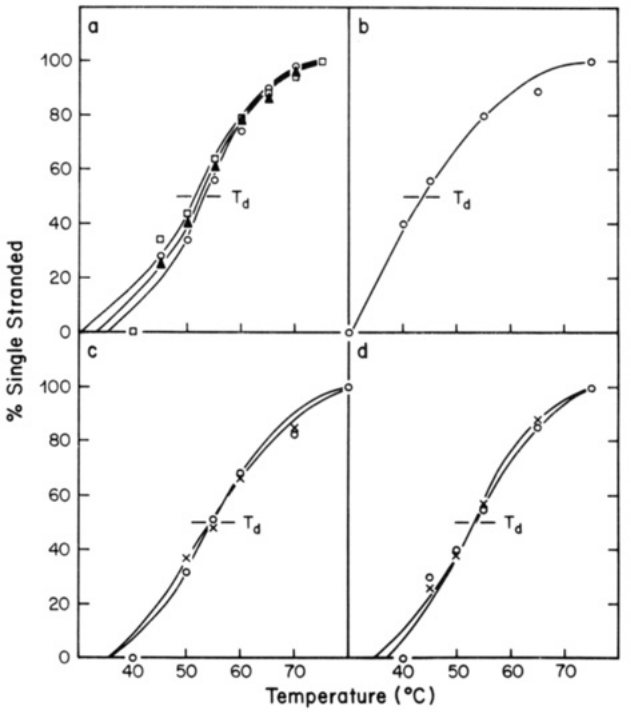

FIGURE 4: Specificity of the oligonucleotide probes determined by heteroduplex dissociation analysis. The melting profiles were determined as described under Experimental Procedures: (a) 3′PSkal$_{21}$; (b) 3′S1kal$_{16}$; (c) 3′S2kal$_{21}$; (d) 5′S3kal$_{21}$. The tissue RNAs used were submaxillary gland (circles), pancreas (squares), kidney (triangles), and prostate gland (×).

mRNA	Hybridization	cpm hybridized	relative conc.
PS	●●●	1143	1.00
S1	● ● ●	251	0.22
S2	●●●	514	0.45
S3	· · ·	103	0.09

FIGURE 5: Relative concentrations of the four kallikrein-related mRNAs in submaxillary gland RNA. Filter-bound polyadenylated RNA in triplicate from rat submaxillary gland was hybridized with the individual ^{32}P-labeled oligonucleotide probes at 5 °C below the experimentally determined T_d for each oligomer. The bound radioactivity was measured by Cerenkov counting after washing and autoradiography. PS, S1, S2, and S3 indicate the kallikrein mRNA probed in each series of dots.

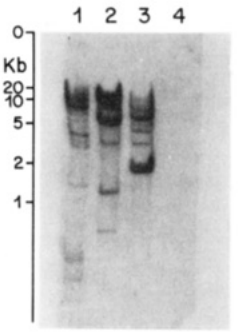

FIGURE 6: Genomic Southern blot analysis of kallikrein-related genes in the rat. Rat liver DNA was digested with *Bam*HI (lane 1), *Eco*RI (lane 2), and *Hin*dIII (lane 3), electrophoresed through an agarose gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled pcXP39 DNA. Lane 4 contained *Hin*dIII-digested λ DNA size markers.

Identification of Multiple Cloned Kallikrein-Related Genes. Molecular cloning of multiple kallikrein-related genes from rat genomic DNA revealed the presence of several genes with

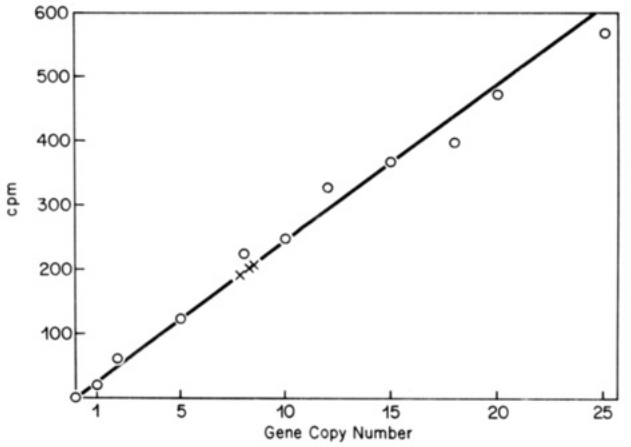

FIGURE 7: Number of kallikrein-related genes in the rat genome. Filter-bound samples of the recombinant kallikrein cDNA plasmid pPS-J or rat liver DNA were hybridized with a single-stranded ^{32}P-labeled kallikrein cDNA probe (see Experimental Procedures). The bound radioactivity was measured by Cerenkov counting after washing and autoradiography.

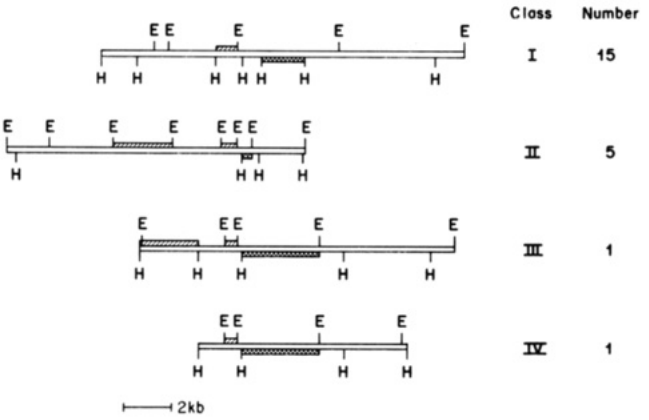

FIGURE 8: Cloned rat genomic fragments bearing kallikrein family genes. The *open rectangles* represent the rat genomic DNA inserts of recombinant λ phage that have been mapped with *Hin*dIII (H) and *Eco*RI (E) restriction endonucleases. The *singly hatched* regions hybridized with pcXP130 (a 5′ end mRNA probe), and the *doubly hatched* regions hybridized with both pcXP130 and pcXP39 (a 3′ end probe). The number of independently isolated recombinant phage for each gene class is indicated.

unique restriction endonuclease maps. A total of 24 recombinant λ phage bearing kallikrein-related gene sequences were isolated from a rat genomic DNA library by screening with cDNA probes for the 5′ and 3′ ends of the PS-type mRNA (see Experimental Procedures). On the basis of restriction endonuclease mapping, the 24 genomic clones can be grouped into 4 classes that represent unique kallikrein-like genes or pseudogenes (Figure 8). Each class hybridized to both the 5′ and 3′ regions of kallikrein mRNA.

The cloned kallikrein-related genes were correlated with two of the four known mRNAs by hybridization of ^{32}P-labeled, mRNA-specific oligonucleotides to Southern blots of *Hin*dIII-digested recombinant λ phage DNA. The 15 class I recombinants have a 1.8-kb *Hin*dIII fragment that hybridized with the 3′PSkal$_{21}$ oligonucleotide (Figure 9a). Therefore, these recombinants represent the gene (or genes) encoding the PS-type kallikrein mRNA. Similarly, the 3′S2kal$_{21}$ probe hybridized to a 0.7-kb *Hin*dIII fragment found in all five members of the class II gene (Figure 9b). The 3′S1kal$_{16}$ and 5′S3kal$_{21}$ oligonucleotides did not hybridize to any of the 24 genomic clones. The class III and IV genes represent members of the kallikrein gene family whose mRNAs have not been identified.

Discussion

To investigate the mechanisms which regulate the expression of individual members of the kallikrein multigene family, it is first necessary to determine the number of active genes as well as their spectrum of tissue-specific expression. Gene copy number analysis of rat nuclear DNA using a PS kallikrein cDNA probe detected approximately 8 kallikrein-related genes, greater than the estimate of 2–3 kallikrein-related genes in the hamster (Howles et al., 1984) but smaller than 25–30 genes in the mouse (Mason et al., 1983). The results of heteroduplex melting temperature experiments indicated that at least five distinct kallikrein-related mRNAs are expressed in the seven rat tissues analyzed.

Specific synthetic oligonucleotides have been used to determine the tissues expressing the four known rat kallikrein-related mRNAs. The most abundant kallikrein mRNA in the submaxillary gland, PS, is also expressed in both the pancreas and kidney, although at lower levels. The partial amino acid sequence of purified rat submaxillary kallikrein determined

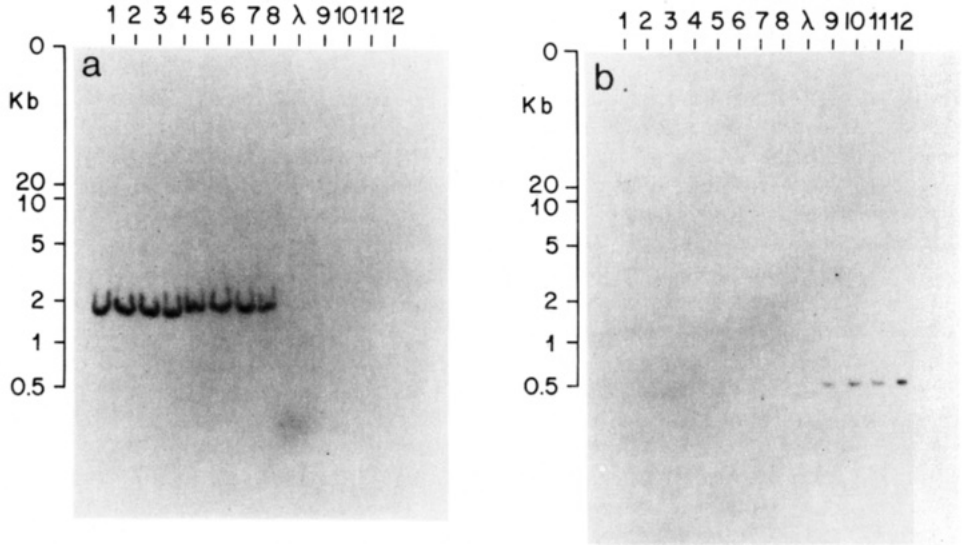

FIGURE 9: Identification of kallikrein-related genomic clones encoding the PS kallikrein and tonin. DNAs of class I and II genomic clones were digested with *Hin*dIII, resolved on agarose gels, transferred to nitrocellulose, and hybridized with 3′PSkal$_{21}$ (a) or 3′S2kal$_{21}$ (b). Lanes 1–8 are class I recombinants; lanes 9–12 are class II recombinants. The lane marked λ contained *Hin*dIII-digested λ phage DNA size markers.

by Lazure et al. (1981) coincides with the amino acid sequence derived from the PS mRNA (Swift et al., 1982). Thus, the PS kallikrein mRNA encodes a kinin-producing enzyme that may function in the regulation of blood flow and electrolyte transport in these tissues (Hilton, 1970). The expression of identical true kallikreins in these three tissues of the rat is consistent with the isolation of identical kallikreins from pancreas, submaxillary gland, and urine of the pig (Fritz et al., 1977).

The amino acid sequence of the enzyme encoded by the S1 kallikrein mRNA is sufficiently similar to that of PS kallikrein to suggest that it has the same substrate cleavage specificity, and therefore the same physiological function. However, its presence specifically in the submaxillary gland and at relatively low levels suggests that it may be involved in the processing of a submaxillary gland specific peptide.

Tonin and the tonin-like enzyme encoded by the S3 mRNA have identical amino acids at key positions thought to be principal determinants of substrate cleavage preferences (Ashley & MacDonald, 1985). Furthermore, among the eight tissues examined, the S2 and S3 mRNAs are expressed in the same two tissues: the submaxillary gland and the prostate. These correlations of common structure and expression suggest a common function as well. However, the role of multiple, secretable tonin enzymes in the physiology of the prostate gland is obscure.

We have isolated at least 4 members of the rat kallikrein gene family; 24 recombinant λ phage from a rat genomic library can be grouped into 4 distinguishable classes on the basis of restriction endonuclease maps. In addition, each recombinant λ clone hybridized to both 5′ and 3′ PS kallikrein mRNA probes, so that each class must represent a different kallikrein-related gene and not simply different ends of the same gene. None of the 24 rat recombinant genomic clones bear more than one kallikrein-like gene, suggesting that the members of the rat kallikrein gene family may be spaced further apart than the analogous mouse gene family members, two of which have been shown to be separated by less than 4 kb (Mason et al., 1983).

The 3′PSkal$_{21}$ oligonucleotide probe has been used to identify the gene(s) encoding the PS kallikrein mRNA. 3′PSkal$_{21}$ hybridized to a 1.8-kb *Hin*dIII fragment common to all members of the class I rat kallikrein genomic clones but did not hybridize to any of the other cloned kallikrein-related genes. The cloned class I gene(s) comprise(s) a subset of 15 recombinant λ phage with identical *Hin*dIII and *Eco*RI restriction maps. The 15 genomic clones were identified by screening only 3 rat genome equivalents of the λ-phage library. In contrast, only five class II recombinants and a single class III and class IV recombinant were isolated. These results suggest that the PS kallikrein gene may exist as multiple copies in the rat genome. In support of this notion, a 1.8-kb *Hin*dIII fragment was the most intensely hybridizing band in genomic Southern blots (Figure 6). Nucleotide sequences of the submaxillary, pancreatic, and kidney PS mRNAs are identical (Ashley & MacDonald, 1985; S. Clift, P. Ashley and R. MacDonald, unpublished results); for the mRNAs in these different tissues to be encoded by different PS genes, the exon sequences of these genes also would have to be identical. Further analysis is required to determine whether the kallikrein multigene family includes multiple identical copies of the PS-type gene, with each copy selectively expressed in a single tissue, or whether a single PS-type gene exists and is expressed in several tissues (e.g., the pancreas, submaxillary gland, and kidney).

The 3′S2kal$_{21}$ oligonucleotide probe for tonin mRNA hybridized selectively to a 0.7-kb *Hin*dIII fragment found in class II genomic clones. Thus, the class II recombinant genomic clones appear to encode tonin. The 0.7-kb *Hin*dIII fragment was barely detectable in genomic Southern blots using a PS cDNA probe, which suggests that the tonin (S2) gene is present at a low copy number (perhaps a single copy) in the rat genome.

The rat kallikrein-related gene family comprises closely related genes encoding physiologically important enzymes. Although the coding domains of the genes are well conserved, the regulated expression of individual family members has diverged. Several of the genes are expressed in discrete subsets of tissues. Further study of the kallikrein-related genes should reveal the general nature of tissue-specific regulatory elements and the variations that direct selective expression to certain tissues. The correlation of kallikrein-related genes with tissue-specific mRNAs will eventually define the limits of expression of the entire kallikrein gene family.

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Quantitative Determination of the 5-(Hydroxymethyl)uracil Moiety in the DNA of γ-Irradiated Cells†

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ABSTRACT: 5-(Hydroxymethyl)uracil (HMUra) is a chemically stable derivative of thymine formed through the action of ionizing radiation which we previously identified in the DNA of γ-irradiated HeLa cells [Teebor, G. W., Frenkel, K., & Goldstein, M. S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 318–321]. In this report, we determine whether HMUra can be used as a marker of exposure of DNA to ionizing radiation. Dose–response curves for its formation in [^{3}H]thymidine-labeled DNA were constructed by exposing the DNA to increasing amounts of γ-radiation and measuring the HMUra content. DNA was irradiated both in solution and in intact cells. HMUra was identified as the 2′-deoxyribonucleoside 5-(hydroxymethyl)-2′-deoxyuridine (HMdU) by subjecting the irradiated DNA to enzymatic digestion and analyzing the mixture of 2′-deoxyribonucleosides by high-pressure liquid chromatography. The identity of the radiogenically formed HMdU was confirmed by acetylation and the structure of the acetyl derivative obtained by mass and nuclear magnetic resonance spectroscopies. At two different DNA concentrations in solution, the same number of thymidine moieties were converted to HMdU, indicating that within this range of concentration the formation of HMdU was mediated through the indirect action of ionizing radiation. Equal amounts of HMdU were formed in single- and double-stranded DNA at each radiation dose, indicating that DNA conformation did not affect HMdU formation. Surprisingly, the *G* value (number of HMdU molecules formed/100 eV) was higher in irradiated cellular DNA than in DNA irradiated in solution. This may be due to the fact that although the thymine moiety in DNA in solution is more vulnerable to hydroxyl radical attack than in the cell, the radiogenically formed 5-methyleneuracil radical re-forms thymine through reaction with hydrogen radicals more readily in solution than in the cell, resulting in a small net yield of HMdU. HMdU was formed in a dose-dependent manner whether the DNA was irradiated in solution or in intact cells. We conclude that HMdU may serve as a quantitative marker of exposure of the genetic material of living cells to ionizing radiation.

The carcinogenic and mutagenic properties of ionizing radiation are thought to be a direct consequence of the damage it causes to DNA (Upton, 1975). Therefore, it is important to find a quantitative marker of radiation damage which could serve in a manner analogous to the cyclobutane pyrimidine dimer induced by UV radiation (Carrier & Setlow, 1971). The damage to DNA effected by ionizing radiation is heterogeneous and includes single- and double-strand breaks, DNA–protein cross-links, loss of bases from the DNA backbone, and chemical modification of the bases remaining on the DNA backbone (Rhaese & Freese, 1968; Dunlap & Cerutti, 1975;

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