overall yield (based on deoxycytidine) after purification was about 30%. After evaporation of solvent (with ethanol to decompose TEAB, and repeated coevaporation with pyridine), a saturated solution of the dry product in pyridine was prepared (350 μmol of monomer in pyridine to a total volume of approximately 0.5 ml). Oligomerization was achieved by addition of 3.5 mmol of dry, finely powdered iodine, which activates the phosphorothioate group by oxidation (*10, 12*). The mixture was agitated in an ultrasonic bath for 1 hour and allowed to stand overnight.

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13. Hydrolysis was performed in a mixture of CH_3OH and 25% NH_4OH (1:1, for 24 hours at 50°C).
14. A preliminary purification was carried out on Q-Sepharose (Pharmacia) by eluting with 0.05*M* TEAB, to bring off nonnucleotide reaction products and cyclic pyrophosphate, and then with 2*M* TEAB. After evaporation of the solvent, the extent of oligomerization was determined by HPLC on RPC-5 (*1*). The products were then separated on Q-Sepharose in a linear gradient of 0.05*M* to 0.6*M* TEAB (*p*H 7.5), which brought off products with chain lengths less than 4. Longer oligomers were recovered in 2*M* TEAB, and this fraction was treated with *Escherichia coli* alkaline phosphatase to remove terminal phosphate groups. For each optical density unit of oligomer, digestion was carried out with 0.1 unit of enzyme (Sigma Type III) in 0.04*M* tris-HCl containing 0.02*M* $MgCl_2$ (*p*H 8) for 4 hours at 37°C. The oligomers were then refractionated on Q-Sepharose in a linear gradient of 0.05*M* to 1.5*M* TEAB.
15. The chemical shift relative to phosphoric acid was −9.5 ppm.
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Access to a Messenger RNA Sequence or Its Protein Product Is Not Limited by Tissue or Species Specificity

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RNA amplification with transcript sequencing (RAWTS) is a rapid and sensitive method of direct sequencing that involves complementary DNA synthesis, polymerase chain reaction (PCR) with a primer or primers containing a phage promoter, transcription from the phage promoter, and reverse transcriptase–mediated sequencing. By means of RAWTS, it was possible to sequence each of four tissue-specific human messenger RNAs (blue pigment, factor IX, phenylalanine hydroxylase, and tyrosine hydroxylase) in four cell types examined (white blood cells, liver, K562 erythroleukemia cells, and chorionic villus cells). These results indicate that there is a basal rate of transcription, splicing, and polyadenylation of tissue-specific mRNAs in adult and embryonic tissues. In addition to revealing sequence information, it is possible to generate a desired in vitro translation product by incorporating a translation initiation signal into the appropriate PCR primer. RAWTS can be used to obtain novel mRNA sequence information from other species as illustrated with a segment of the catalytic domain of factor IX. In general, the ability to obtain mRNA sequences rapidly across species boundaries should aid both the study of protein evolution and the identification of sequences crucial for protein structure and function.

RECENTLY, METHODS HAVE BEEN described for the direct sequencing of genomic DNA that are based on PCR (*1, 2*). One of these methods, known as genomic amplification with transcript sequencing (GAWTS), incorporates a phage promoter sequence into at least one of the PCR primers (*2*). GAWTS has been modified to allow RNA to be directly sequenced. RAWTS consists of four steps: (i) cDNA synthesis with oligo(dT) or an mRNA-specific oligonucleotide primer, (ii) PCR where one or both oligonucleotides contains a phage promoter attached to a sequence complementary to the region to be amplified, (iii) transcription with a phage promoter, and (iv) reverse transcriptase–mediated dideoxy sequencing of the transcript, which is primed with a nested (internal) oligonucleotide. The incorporation of a phage promoter by PCR has three major advantages: (i) transcription produces a second round of amplification, which obviates the need for purification subsequent to PCR; (ii) transcription can compensate for suboptimal PCR; and (iii) transcription generates a single-stranded template, which in routine practice tends to give a more reproducible sequence than obtained directly from a linear double-stranded PCR product.

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RAWTS is extraordinarily sensitive because it combines the amplification generated by phage transcription with the amplification generated by PCR. To determine whether tissue-specific mRNAs could also be detected, total RNA was isolated from white blood cells, liver, K562 erythroleukemia cells, and cultured chorionic villus cells. The RNA was isolated by lysing the cells in the presence of guanidium-HCl except for the K562 cells, in which lysis occurred into SDS/proteinase K, followed by phenol extraction (*3*). Single-stranded cDNA was made from total RNA by priming reverse transcriptase with oligo(dT). RAWTS was performed on four tissue-specific mRNAs: blue pigment, which is expressed in the retina; factor IX and phenylalanine hydroxylase, which are expressed in the liver; and tyrosine hydroxylase, which is expressed in the brain and adrenal gland. How the primers were chosen for the retina-specific blue pigment mRNA is shown in Fig. 1. The first set of PCR primers were: BP-(T7-29)E5(1453)-44U, which contains the T7 promoter sequence, and BP-E4(1230)-16D [Fig. 1; for an explanation of the notation, see (*4*)]. The primers chosen span at least one intron, so the genomic sequence can be distinguished from that of mRNA. Sequence of the blue pigment mRNA was not obtained from white blood cell RNA even after 40 cycles of PCR. Therefore, we did a second amplification by diluting an aliquot of the first PCR mix 1000-fold in a fresh PCR mix and reamplifying with the same T7 promoter oligonucleotide primer along with E4(1259)-17D. An amplified fragment of predicted size was seen (Fig. 2A). The fragment was transcribed and then sequenced with BP-E4(1293)-17D as the primer for reverse transcriptasse. Intronic sequence was absent, confirming that mRNA and not DNA was the origin of the signal (Fig. 2C). By performing the two rounds of PCR, sequence information could also be obtained from liver, K562, and chorionic villus RNA (Fig. 2A and Table 1). With one

Table 1. Sequencing of tissue-specific human mRNAs. Blue pigment, BP; factor IX, F9; phenylalanine hyroxylase, PH; tyrosine hyroxylase, TH.

Tissue	BP	F9	PH	TH
Blood	+	+	+	+
Liver	+	+	+	+
K562	+	+	+	+
CVS	+	?*	+	+

*Factor IX could not be amplified from cultured chorionic villus cells but this is most likely a consequence of the partial degradation of this RNA (as indicated by the relative intensity of the 28*S* and 18*S* ribosomal RNA species), since the primers utilized for amplification required that more than 2 kb of the mRNA be intact, whereas sequencing of the other mRNAs required that no more than 1.1 kb be intact.

or more rounds of PCR for which one or both primers differed in the second round, the other three tissue-specific mRNAs were also sequenced so as to confirm the absence of introns by the use of the primers described in the legend to Fig. 2 (Fig. 2, B and C, and Table 1). Despite the extensive amplification, no sequencing errors were found in 3 kb of sequence (15 combinations of mRNAs and cells at 150 to 250 bases per combination). This is expected because direct sequencing provides data on a population of molecules rather than a clone of one molecule. So long as multiple mRNA molecules are amplified on the first round of PCR, an error at a specific base during polymerization will not materially affect the predominant sequence of the population of molecules.

The great sensitivity of PCR can potentially lead to artifact. However, the mRNA sequence that was obtained cannot be due to plasmid contamination because, with the exception of the factor IX gene, cloned sequences of these genes were not present in the laboratory. Likewise, no retina, brain, or adrenal mRNA was present in the laboratory. In addition, contamination of solutions with previously amplified material was routinely monitored by verifying that no segments were seen when PCR was performed without input cDNA. The unique sequence from factor IX mRNAs of other species also demonstrates the veracity of the sequence (see below). Finally, the possibility of processed pseudogenes accounting for the data is eliminated by previously published data (*5–10*) and the results of amplification of genomic DNA for these genes (that is, no amplified segment is seen at the size expected for mRNA).

These data indicate that some level of mRNA synthesis occurs for at least some and possibly even all tissue-specific genes. Since one round of PCR followed by transcription can amplify a segment one billion times, two rounds of PCR should detect mRNAs that are present at very much less than one copy per cell. The levels of these mRNAs in various tissues are of interest, but precise quantitation depends on (i) quantitative isolation of RNA, which is difficult in tissues with very active ribonucleases such as in blood; (ii) measurement of the efficiency of cDNA synthesis, which depends on multiple parameters including the concentrations of RNA, reverse transcriptase, and primers as well as the particular size and sequence of the mRNA of interest; and (iii) measurement of the efficiency of PCR, a reaction in which small differences in efficiency per cycle are exponentially amplified.

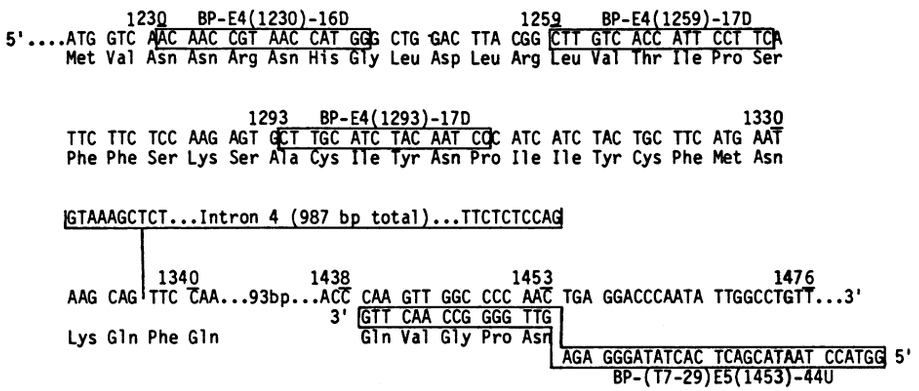

Fig. 1. Oligonucleotides for RAWTS of a segment of blue pigment mRNA. Shown are portions of exons 4 and 5, which correspond to the region that was amplified and sequenced. The numbering system of Nathans *et al.* (*6*) was used. The first PCR was performed with the oligonucleotide primers BP-E4(1230)-16D and BP-(T7-29)E5(1453)-44U [for an explanation of the notation, see (*4*)]. A 253-bp amplified segment would be expected from blue pigment mRNA (224 bp of blue pigment sequence and a 29-bp T7 promoter sequence), and a 1240-bp amplified fragment is expected from genomic DNA. The 253-bp segment was not seen, so reamplification was performed with BP-E4(1259)-17D and BP-(T7-29)E5(1453)-44U. The expected 224-bp segment was generated and subsequently transcribed. Dideoxy sequencing was then performed by using BP-E4(1293)-17D as the internal primer for reverse transcriptase [see methods in (*18*)].

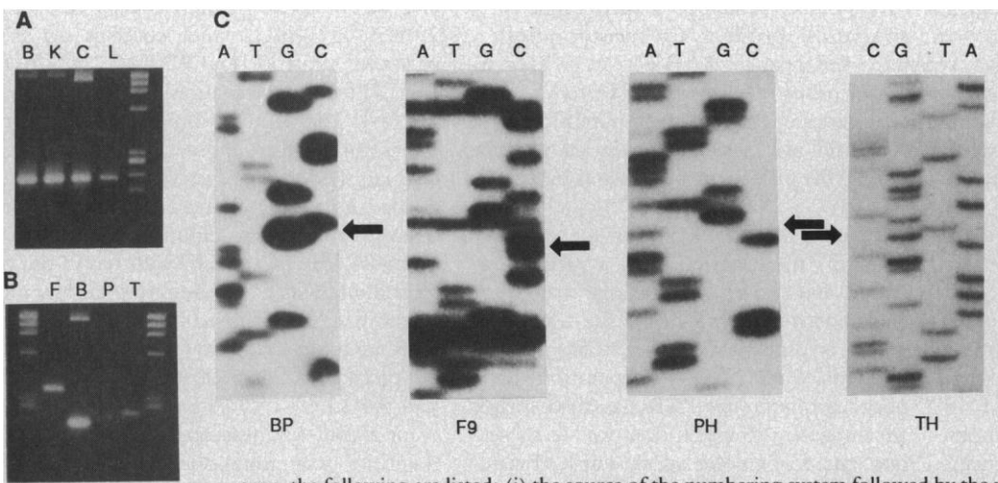

Fig. 2. RAWTS of tissue-specific mRNA. (**A**) Ethidium bromide stain of 2.5% agarose gel subsequent to two rounds of PCR of the blue pigment gene (see Fig. 1 and text) from 100 ng of total RNA extracted from blood, K562 cells, chorionic villus cells, and liver (lanes B, K, C, and L, respectively). The rightmost lane shows size standards produced by Hae III digestion of ϕX174. (**B**) Agarose gel of PCR amplification of factor IX, blue pigment, phenylalanine hydroxylase, and tyrosine hydroxylase mRNA from total RNA extracted from blood (lanes F, B, P, and T, respectively). In some cases, the amplification shown was the result of more than one round of PCR (for example, see Fig. 1). Each amplified segment spanned an intron. For each gene, the following are listed: (i) the source of the numbering system followed by the reference (if different) detailing the position of the introns, (ii) the PCR primers [for explanation of notation, see (*4*)], and (iii) the expected size of the segments of the mRNA and genomic DNA, which includes the 29 bp of the T7 promoter. Factor XI: (i) Yoshitake *et al.* (*5*); (ii) F9-(T7-29)E7(30057)-46D and F9-E8(31047)-15U; (iii) 351 bp for RNA and 1019 bp for DNA. Blue pigment: (i) Nathans *et al.* (*6*); (ii) PCR 1: BP-E4(1230)-16D and BP-E5(1453)-44U; PCR 2: BP-E4(1259)-17D and BP-E5(1453)-44U; (iii) for PCR 2, 224 bp for mRNA and 1111 bp for DNA. Phenylalanine hydroxylase: (i) Kwok *et al.* (*7*), DiLella *et al.* (*8*); (ii) PH-(T7-29)E13(1626)-46U and PH-E12(1420)-16D; (iii) 235 bp for RNA and ~1400 bp for DNA. Tyrosine hydroxylase: (i) Grima *et al.* (*9*), O'Malley *et al.* (*10*); (ii) PCR 1: TH-E8(936)-15D and TH-E13(1507)-15U, after 100,000-fold dilution, PCR 2: Th-(T7-29)E11(1111)-49D and TH-E13(1507)-15U, after 100,000-fold dilution, PCR 3: TH-(T7-29)E11(1111)-49D and TH-E12(1333)-16U; (iii) 251 bp for RNA and 471 bp for DNA. (**C**) Sequence of the exon intron junction of blue pigment (BP), factor IX (F9), phenylalanine hydroxylase (PH), and tyrosine hydroxylase (TH) mRNA from blood, which verifies that intronic sequence is absent. For methods, see (*18*).

The ability to detect basal levels of tissue-specific mRNA has certain practical consequences as illustrated with the factor IX gene. First, the exonic sequence for an individual with hemophilia B can be obtained from DNA, but doing so requires multiple amplifications because the eight exons of the factor IX genes are dispersed over 34 kb of genomic DNA. Given the current limits on the size of efficiently amplified fragments (*11*), six regions must be amplified and transcribed to sequence the 1383 bp of the coding region. In contrast, the entire sequence of the coding region may well be obtained from RNA with only one amplification and transcription. Second, the consequences of mutations such as the hemophilia-related point mutation at the splice donor junction of intron f (*12*) may well be delineated without exposing the patient to a liver biopsy, a procedure whose hazards cannot generally be justified by a desire to analyze the structure of mRNA.

Once an mRNA segment has been amplified distal to a phage promoter, it is also possible to obtain the protein product by in vitro translation or by insertion into an appropriate expression vector. For factor IX, the carboxyl-terminal 287 amino acids encoded by exons f through h were made by RNA amplification with in vitro translation (RAWIT). An eight-nucleotide translation initiation signal [CCACCATG (*13*)] was added 3′ to the T7 promoter sequence of the PCR primer. When the PCR product was transcribed in the presence of 7mGpppG (*14*), a capped RNA was generated, which contained a predicted 5′ untranslated leader of only 11 bases and a 3′ untranslated region of 146 bases. The capped RNA specifically produced a peptide of expected size in both a reticulocyte (Fig. 3) and a wheat germ lysate (*15*). RAWIT performed with alternate PCR primers yielded similar amounts of peptides of predicted size despite the absence of a termination codon and all 3′ untranslated sequences (Fig. 3). The ability to produce a desired segment of a protein rapidly by RAWIT should facilitate the delineation of relations between structure and function.

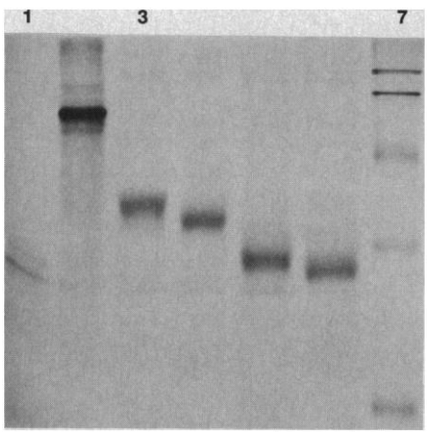

Fig. 3. RNA amplification with in vitro translation (RAWIT). E5(20365)-51D [full name: F9(Hs)-(T7/TI-37) E5(20365)-51D] has a sequence: GGATCCTAATACGACTCACTATAGGGAGA CCACCATG CCATTTCCATGTGG. It contains a 29-base T7 promoter sequence followed by an 8-base translation initiation signal and a 14-base sequence complementary to exon f. PCR was performed with E5(20365)-51D and one of four additional oligonucleotides. The transcript produced by T7 contains an 11-nucleotide leader (GGGAGACCACC) followed by the initiating ATG in frame with the coding sequence. Lane 1, in vitro translation without an RNA template; lane 2, in vitro translation product of full-length factor IX mRNA; lane 3, in vitro translation product of the transcript derived from a PCR for which we used E6(20365)-51D and E8(31515)-16D. The transcript produced with T7 RNA polymerase codes for an 11-base 5′ untranslated region, a 288–amino acid peptide of predicted molecular weight 31,486 and a 146-base 3′ untranslated segment. Lanes 4 to 6, in vitro translation product of a transcript derived from a PCR for which we used E6(20365)-51D plus E8(31330)-16U, E8(31215)-16U, and E8(31189)-16U, respectively. The predicted molecular weights of the peptides are 28,785, 25,872, and 25,124, respectively. Lane 7, protein size markers (Amersham): 92.5, 69, 46, 30, and 14.3 kD, respectively. For each in vitro translation, the peptide of predicted size was seen.

	201							
human		KVDAF	CGGSI	VNEKW	IVTAA	HCVET	GVKIT	VVAGE
mouse		EIE	A	I		LKP	D E	
rat		EIE	A	I		LKP	D E	
guinea pig		ETE				IL	I E	K
rabbit		E		I	V	IKP	DDN	
sheep		EIA			V	IKP		
cow		EIA			V	IKP		

HNIEE	TEHTE	QKRNV	IRIIP	HHNYN	260
Y DK	K D	R	T	Q	
D	K D	R	T	Q	
K	K D	R	TQ L	S	
Q	N			Y K	
K	P P		A	Y G	
T K	P P		A	Y S	

Fig. 4. Cross-species sequencing with RAWTS (ZooRAWTS). Novel amino acid sequences of amino acids 201 to 260 of the factor IX gene of mouse, rat, guinea pig, rabbit, and sheep were obtained by performing RAWTS on mRNA from liver. The liver mRNAs were purchased from Clontech. Complementary DNA was generated with oligo(dT) (*18*) and then PCR was performed under low stringency (increased magnesium concentration) with the human primers (T7-29)E7(30057)-46D and E8(31048)-15U. An amplified segment of expected size was obtained from mouse and rat liver cDNA. Sequence was obtained by using E8(31048)-15U as the primer for reverse transcriptase, but the use of a PCR primer for sequencing did not produce data of uniformly high quality. From those data, an oligonucleotide complementary to both mouse and rat factor IX was synthesized and then used as a nested sequencing primer. This resulted in sequence data without any ambiguities. For guinea pig, rabbit, and sheep, a different pair of human primers was used to obtain the initial sequence. Then a sheep-specific primer was synthesized and successfully utilized to obtain sequence data from the three species. The previously determined amino acid sequence of the corresponding region of bovine factor IX is included for completeness (*19*). The DNA sequence is available from the authors on request.

In addition to sequencing and translating mRNA from different tissues, it would be useful to rapidly determine mRNA sequence in other species. The human factor IX PCR primers (T7-29)E7(30057)-46D and E8(31048)-15U were used to amplify a cDNA segment derived from mouse and rat liver mRNA. A series of amplifications were performed with increasing amounts of magnesium in order to decrease the stringency of annealing. Segments of the expected size were seen at 5 m*M* $MgCl_2$ in both mouse and rat. Attempts to sequence the rodent fragments by using the internal human oligonucleotide were unsuccessful. However, the PCR primer could be used to generate a sequence, albeit of low quality. From this sequence, a mouse-specific oligonucleotide was designed, and resequencing of the transcript with this primer gave high-quality sequence information for both mouse and rat (Fig. 4). By using an alternate pair of PCR primers, it was possible to obtain high-quality sequences for guinea pig, rabbit, and sheep (Fig. 4). A comparison of nucleotide and amino acid sequences indicates that this segment of the catalytic domain of factor IX has evolved at approximately an average rate (*16*). However, the loop of amino acids formed by a postulated disulfide bond at Cys^{206} and Cys^{222} (*5*) is highly conserved. Since His^{221} is known to participate in the catalytic reaction (*5*), we speculate that this loop is important for the formation of the active site.

Analysis of sequences from multiple species is helpful in interpreting changes found in hemophiliacs. As examples, Cys^{222} and Asn^{260} are conserved in all species examined, providing further evidence that the substitutions at these positions found in a severe hemophiliac (factor IX coagulation = 1%), and a mild hemophiliac (factor IX coagulant = 24%), respectively, represent the causative mutations rather than rare polymorphisms (*17*).

In summary, incorporation of a phage promoter into a PCR oligonucleotide primer allows an abundance of transcript to be made after amplification of mRNA by PCR. The sensitivity of the technique allows tis-

sue-specific mRNAs to be sequenced from at least some and possibly all tissues, and the conservation of sequence through evolution allows mRNAs from other species to be sequenced without cloning. In addition, the transcript can be translated in vitro, thereby allowing the intact protein or any desired segment to be produced.

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18. RAWTS is a four-step procedure. (i) *First strand cDNA synthesis:* 20 λ of 50 μg of heat-denatured total RNA or mRNA per milliliter, 50 m*M* tris-HCl (*p*H 8.3), 8 m*M* $MgCl_2$, 30 m*M* KCl, 1 m*M* dithiothreitol (DTT), 2 m*M* each of dATP, dCTP, dGTP, dTTP, oligo(dT) 12–18 (50 μg/ml), RNasin (1000 U/ml), and AMV reverse transcriptase (1000 U/ml) were incubated at 42°C for 1 hour followed by 65°C for 10 min. Subsequently, 30 λ of H_2O was added for a final volume of 50 λ. (ii) *PCR:* 1 λ of the above sample was added to 40 λ of 50 m*M* KCl, 10 m*M* tris-HCl (*p*H 8.3), 1.0 to 2.5 m*M* $MgCl_2$ (empirically determined for each set of primers), 0.01% (w/v) gelatin, 200 μ*M* each of dNTP, 1 μ*M* of each primer (Perkin-Elmer Cetus protocol). After 10 min at 94°C, 1 U of *Taq* polymerase was added, and 40 cycles of PCR were performed (annealing: 2 min at 50°C; elongation: 3 min at 72°C; denaturation: 1 min at 94°C) with the Perkin-Elmer Cetus automated thermal cycler. One primer included a T7 promoter as previously described (*2*). (iii) *Trancription:* After a final 10-min elongation, 3 λ of the amplified material was added to 17 λ of the RNA transcription mixture. The final mixture contains 40 m*M* tris-HCl (*p*H 7.5), 6 m*M* $MgCl_2$, 2 m*M* spermidine, 10 m*M* sodium chloride, 0.5 m*M* of the four ribonucleoside triphosphates, RNasin (1 U/λ), 10 m*M* DTT, 10 U of T7 RNA polymerase, and diethylpyrocarbonate-treated H_2O. Samples were incubated for 1 hour at 37°C, and the reaction was stopped by heating at 65°C for 10 min. (iv) *Sequencing:* 2 λ of the transcription reaction was added to 10 λ of annealing buffer containing the end-labeled reverse transcriptase primer. Annealing and sequencing were performed essentially as described, but note that [δ-^{32}P]ATP is the correct donor for end-labeling (*2*).
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Expression of High-Affinity Binding of Human Immunoglobulin E by Transfected Cells

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The receptor with high affinity for immunoglobulin E (IgE) on mast cells and basophils is critical in initiating allergic reactions. It is composed of an IgE-binding α subunit, a β subunit, and two γ subunits. The human α subunit was expressed on transfected cells in the presence of rat β and γ subunits or in the presence of the γ subunit alone. The IgE binding properties of the expressed human α were characteristic of receptors on normal human cells. These results now permit a systematic analysis of human IgE binding and a search for therapeutically useful inhibitors of that binding.

THE HIGH-AFFINITY RECEPTOR FOR IgE ($Fc_\epsilon RI$) plays a central role in initiating allergic reactions by coupling the presence of allergen to the release of mediators by mast cells and basophils. Using basophilic leukemia (RBL) cells, we showed that $Fc_\epsilon RI$ in the rat is a tetramer consisting of a single IgE-binding α subunit, a single β subunit, and two disulfide-linked γ subunits (*1*). Little is known about the human $Fc_\epsilon RI$ because obtaining sufficient numbers of normal mast cells and basophils is impractical and because no stable cell lines expressing high-affinity receptors for human IgE exist as yet. Knowledge about the structure and function of the human receptor will be critical for determining whether this protein can serve as an appropriate target for new therapeutic approaches to allergy, for example, by inhibiting IgE binding or the earliest steps in receptor activation.

Our aim therefore was to obtain expression of human IgE binding by transfected cells. The cDNA for the IgE-binding human α subunit has been cloned but could not be expressed in transfection experiments (*2, 3*). Possibly, as with the homologous rat α, expression requires cotransfection with the cDNA for the β (*4*) and γ subunits (*5*). However, the library from which the cDNA for human α was obtained has not yet yielded cDNA clones for human β and γ (*6*), and indeed the cell line used to construct that library fails to bind IgE (*7*). We therefore attempted to express human IgE binding by cotransfecting cells with the cDNA for the human α subunit together with the cDNA for rat β or γ subunits, or both.

A 907-bp Sfa NI fragment was excised from the cDNA that codes for the α subunit of the human IgE receptor and subcloned into the vector pSVL for expression by transfected COS 7 cells. This fragment includes the entire sequence coding for α plus a minimal amount of 5′ and 3′ untranslated sequence. The pSVL–human α cDNA was cotransfected along with separate pSVL plasmids containing cDNAs coding for the rat β and γ subunits. When these transfectants [$\alpha_H(\beta\gamma)_R$ cells] were reacted with mouse monoclonal IgE (specific for dinitrophenyl), 5% to 7% of cells formed rosettes with trinitrophenylated red blood cells. Cells cotransfected with α_H and β_R failed to form rosettes, but $\alpha_H\gamma_R$ transfectants showed rosetting (Table 1). The latter result was unexpected because we had found earli-

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