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# Expression of Human Angiogenin in Cultured Baby Hamster Kidney Cells†

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ABSTRACT: Baby hamster kidney cells were transformed with DNA sequences derived from the gene for human angiogenin. Expression was under the transcriptional control of the inducible mouse metallothionein 1 promoter. Recombinant angiogenin was purified and shown to be chemically, biologically, and enzymatically indistinguishable from the natural product. The large-scale production of recombinant angiogenin achieved should facilitate detailed studies into the structure–function relationships of this potent angiogenic molecule.

Angiogenin, a single-chain cationic polypeptide of $M_r$ 14124, is a potent stimulator of blood vessel formation in both the chick chorioallantoic membrane and rabbit cornea (Fett et al., 1985). Originally isolated from medium conditioned by human colon adenocarcinoma cells (Fett et al., 1985), angiogenin has recently been detected in and isolated from normal human plasma (Shapiro et al., 1987). Thirty-five percent of its sequence is identical with that of human pancreatic ribonuclease (Strydom et al., 1985), and it, in fact, exhibits ribonucleolytic activity which is characteristic of and differs distinctly from that of pancreatic ribonuclease (Shapiro et al., 1986). The chemical structures of its gene and cDNA, isolated from normal liver libraries, have been determined (Kurachi et al., 1985).

Until recently there has not been enough purified angiogenin to carry out detailed investigations of its physiological mechanisms, interactions with substrates and/or target cells and tissues, and antigenic properties as well as potential clinical applications. In order to obtain sufficient material necessary for such studies, mammalian cell expression systems were investigated. We here report the successful engineering of such

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a system resulting in high-level expression of angiogenin that is indistinguishable from the native product by multiple criteria.

## Experimental Procedures

*General.* Restriction endonucleases, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were purchased from Bethesda Research Laboratories or Boehringer Mannheim. Endonuclease *Bal*31 was from International Biotechnologies, Inc. Plasmid pBR322–*Sma*I was from Pharmacia. Plasmid pUC13 and cloning vector M13mp18 were from Bethesda Research Laboratories.

Large-scale preparations of plasmid DNA were obtained by centrifugation on 5.7 M CsCl, and small-scale DNA preparations for rapid analysis were performed by the alkaline lysis method described by Maniatis et al. (1982). Fragments of DNA were analyzed and purified by agarose gel electrophoresis (Maniatis et al., 1982).

Plasmid pMTFIX, a vector containing the gene for human factor IX, the inducible mouse metallothionein 1 (MT-1) promoter, the SV40 late promoter, and a modified DHFR[1] gene for selection purposes, was kindly provided by Dr. Richard Palmiter (Kurachi & Palmiter, 1985).

*Construction of Expression Vectors.* The ~5-kilobase *Pvu*II fragment containing the angiogenin gene (λHAG1; Kurachi et al., 1985) cloned into a derivative of pBR322 at a unique *Sma*I site was used as starting material (Figure 1). The resultant plasmid (pBR322–HAGF) was linearized with *Bgl*II, which cleaved uniquely in the 5′ untranslated region of the HAGF sequence. The linearized plasmid was then digested with *Bal*31 to remove nucleotides upstream of the coding sequence. Aliquots of the reaction mixture were removed at 10, 30, 45, and 60 s and subjected to digestion with *Bam*HI. The resulting fragment ends were blunted by using the large fragment of DNA polymerase I (Klenow fragment) in the presence of 0.2 mM deoxynucleotide triphosphates. The fragment containing the pBR322 and HAGF coding sequence was separated by electrophoresis on a 0.7% agarose gel, extracted from the gel, and recircularized.

The plasmid was then transfected into *Escherichia coli* (strain RR1), and DNA isolated from the resulting colonies was subjected to restriction endonuclease analysis with *Bam*HI and *Eco*RI to determine which clones had regenerated the *Bam*HI site. Appropriate clones were amplified, and the DNA obtained was digested with *Bam*HI and *Eco*RI in order to recover the insert DNA containing the angiogenin sequence. A portion of the recovered inserts was ligated to M13mp18 and sequenced to determine the 5′-end start site of the insert DNA while the remainder was ligated to pUC13 for amplification.

The expression vector containing the angiogenin gene was then constructed by employing plasmid pMTFIX. This plasmid was digested with *Bam*HI and *Eco*RI, and the fragment comprising the pUC sequence and the MT1–SV40–DHFR expression unit was separated from the human factor IX coding sequence by agarose gel electrophoresis. The purified vector (pMT) was then ligated to the angiogenin gene construct, which contained the entire gene and ~2 kilobases of the 3′ flanking sequence similarly obtained by *Bam*HI–*Eco*RI digestion and agarose gel electrophoresis. Among many resultant expression vector constructs, one that contains the angiogenin gene beginning at +7 from the site of transcription initiation (Kurachi et al., 1985) was designated pMTAG(+7) (Figure 1). A second construct with the angiogenin gene starting at +64, designated pMTAG(+64), was constructed by modifying pMTAG(+7) as follows. An aliquot (20 μg) of pMTAG(+7) was digested with *Bam*HI. The digested plasmid DNAs were then purified by phenol extraction and subjected to *Bal*31 digestion. At 0.5, 1, 2, and 3 min aliquots of the reaction mixture were removed into phenol and immediately vortexed. DNAs recovered were then blunt-ended by using the Klenow fragment of DNA polymerase I and ligated to *Bam*HI linkers according to standard methods (Maniatis et al., 1982). DNA samples were then digested with *Bam*HI and *Eco*RI, followed by electrophoresis on 0.8% agarose. Angiogenin gene inserts of various sizes with *Bam*HI and *Eco*RI sticky ends were recovered from the gel and ligated to pMT that did not contain the angiogenin gene insert. This vector was previously prepared from pMTAG(+7) by digestion with *Bam*HI and *Eco*RI. The plasmid DNAs were then used to transform *E. coli*. Minipreparations of the plasmid DNAs from the recombinant bacteria were prepared, and inserts released by digestion with *Bam*HI and *Eco*RI were analyzed for size. Selected inserts were also subjected to DNA sequencing. Plasmid pMTAG(+64), which contained an angiogenin insert beginning at +64, was selected as the second construct to be investigated for expression since it lacks the potential translation initiation sequence beginning at +52, which is present in pMTAG(+7).

*Transfections and Cell Culture.* BHK cells (kindly provided by Dr. Richard Palmiter) were routinely propagated by using DME (M. A. Bioproducts) supplemented with 4–5 g/L glucose, 50 mg/L gentamicin, 500 μg/L fungizone, 2 mM L-glutamine, and 10% heat-inactivated calf serum (DME/10). Cultures were maintained at 37 °C in a 7.5% $CO_2$ in air environment.

Transfections were performed by the conventional calcium phosphate precipitation technique (Graham & Van der Eb, 1973). For selection of transfectants, cells were cultured sequentially in DME/10 that contained increasing concentrations of MTX (1, 10, and 100 μM, 1 mM). Colonies surviving in 1 mM MTX were expanded and induced with 80 μM $ZnSO_4$ and 2 μM $CdSO_4$, separately or in combination, for assessment of angiogenin mRNA and protein levels. Mixed cultures found to secrete suitable amounts of angiogenin were cloned by limiting dilution.

Large-scale cultures of transfected clones employed 6000-$cm^2$ Nunc multilevel cell factories (Vangard International). Briefly, $1 \times 10^8$ cells were inoculated into a cell factory containing 2.0 L of DME/10 and allowed to attach and proliferate in a humidified, 7.5% $CO_2$ in air environment at 37 °C until confluent. The DME/10 was then replaced with 2.0 L of DME/10 containing 80 μM $ZnSO_4$ and 2 μM $CdSO_4$. This induction medium was changed every 2–3 days. At approximately weekly intervals cells were incubated for 2 days in DME/10 to recover from metal exposure and then again placed in metal-supplemented medium. Conditioned medium was collected and processed as described below.

*Solution Hybridization.* Angiogenin mRNA was assayed essentially as described by Durnam and Palmiter (1983). A synthetic oligonucleotide (20-mer) complementary to the coding sequence for amino acids 35–41 of angiogenin was labeled with $^{32}P$ at its 5′-end. Total nucleic acids were prepared by homogenization, phenol/chloroform extraction, and

[1] Abbreviations: BHK, baby hamster kidney; CAM, chorioallantoic membrane; DHFR, dihydrofolate reductase; DME, Dulbecco's modified Eagle's medium; HPLC, high-performance liquid chromatography; MTX, methotrexate; PAGE, polyacrylamide gel electrophoresis; NaDodSO$_4$, sodium dodecyl sulfate; λHAG1, genomic clone (17 kilobases) containing the angiogenin gene; HAGF, ~5-kilobase *Pvu*II fragment of λHAG1 containing the angiogenin gene.

ethanol precipitation (Durnam & Palmiter, 1983). Approximately $^1/_{10}$ of the total RNA prepared from $1 \times 10^6$ BHK cells was hybridized to the 5′-end labeled probe. After an overnight incubation, the reaction mixture was treated with S1 nuclease (8 units) for 1 h, followed by precipitation of the protected mRNA by 5% trichloroacetic acid. The samples were then filtered onto Whatman GF/C filters, washed 3 times with cold 5% trichloroacetic acid and once with 70% ethanol, dried, added to 2 mL of toluene/Omnifluor scintillation fluid, and counted. A standard curve was constructed by using known amounts of sense-strand DNA from an M13mp18 clone containing the coding region of the angiogenin gene.

*Northern Analysis.* Total cellular RNA was isolated from BHK cells by the guanidinium thiocyanate method and purified by sedimentation through 5.7 M CsCl (Chirgwin et al., 1979). Poly(A)-enriched fractions were prepared by oligo(dT)–cellulose chromatography (Aviv & Leder, 1972).

A probe consisting of the 697 base pair angiogenin cDNA insert was generated by nick translation (Rigby et al., 1977) to a specific activity of $3 \times 10^8$ cpm/$\mu$g. Total RNA (15 $\mu$g) was size fractionated through a 1.2% denaturing formaldehyde agarose gel (Lehrach et al., 1977). Following electrophoresis, the RNA was blotted onto nitrocellulose filters (Thomas, 1980), baked at 80 °C, and hybridized to the $^{32}$P-labeled probe in buffer containing 50% formamide at 42 °C. The filters were washed 3 times for 2 h at 65 °C in 0.075 M sodium chloride/0.01 M sodium citrate, pH 7.0, and exposed to Kodak XAR-5 film overnight.

*Purification of Angiogenin.* Recombinant angiogenin was isolated from conditioned medium by a modification of procedures described previously (Fett et al., 1985; Shapiro et al., 1986, 1987). Briefly, for initial small-scale preparations, medium (up to 1 L) was brought to pH 6.6, supplemented with 1 mg of hen egg white lysozyme (Sigma), and chromatographed on a column of CM-52 cation-exchange resin (Fett et al., 1985). Material eluting with 1 M NaCl was then applied directly to a Synchropak RP-P C18 HPLC column (250 × 4.1 mm; Synchrom, Inc.). Elution was achieved with a 45-min linear gradient from 30% to 40% solvent B where solvent A is 0.1% TFA in water (v/v) and solvent B is 2-propanol/acetonitrile/water (3:2:2 v/v) with 0.08% TFA. The flow rate was 1.0 mL/min, and 1-min fractions were collected. A Waters Associates liquid chromatography system was employed. For large-scale preparations, medium (10–30 L) was chromatographed on CM-52 as for human plasma (Shapiro et al., 1987). Material eluting with 1 M NaCl was concentrated by ultrafiltration with a YM-5 membrane (Amicon), clarified by centrifugation at 15600*g*, and then diluted 7-fold with 10 mM Tris, pH 8.0. The resultant suspension was then injected onto a Mono S cation-exchange column (50 × 5 mm, Pharmacia) and angiogenin eluted as described (Shapiro et al., 1987). Angiogenin tested in the CAM assay was obtained by this procedure and was dialyzed vs water prior to use.

Angiogenin preparations tested for ribonucleolytic activity were subjected to additional purification steps in order to remove all traces of contaminating RNases. Material eluting from the C18 column was partially evaporated under nitrogen, supplemented with 150 $\mu$L/mL 0.1 M Tris, pH 8.0, and then chromatographed on the Mono S column. Angiogenin eluting from this column was then rechromatographed on the C18 column as described above.

*Chemical Procedures.* Amino acid analyses, protein and peptide sequence analyses, and generation of peptide fragments and their isolation were performed essentially as detailed previously (Strydom et al., 1985). The Beckman sequencer employed was, however, modified to 890 M status, and Beckman sequencer-grade chemicals were used. The Picotag methodology for amino acid analyses used methanol instead of ethanol as solvent to alleviate problems caused by high salt concentrations in some samples. Tryptophan was determined by the phenyl isothiocyanate method after hydrolysis with 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)-indole (Pierce Chemical Co.) (Liu & Chang, 1971).

*Enzymatic Assays.* Pancreatic RNase-like activity was measured by the production of perchloric acid soluble material from wheat germ RNA as described (Blank & Dekker, 1981; Shapiro et al., 1986). Activity toward 28S and 18S rRNAs was examined by agarose gel electrophoresis followed by ethidium bromide staining (Shapiro et al., 1986).

*Biological Assays.* Angiogenesis was assessed by using the CAM method of Knighton et al. (1977) as described (Fett et al., 1985). The number of eggs employed in any individual set of assays for a given concentration ranged from 5 to 20. Statistical analyses were performed as detailed by Shapiro et al. (1987).

*Immunoblot (Western) Analyses.* $NaDodSO_4$–PAGE was performed in the absence of reducing agents by using 15% gels as described by Laemmli (1970). Gel slabs were washed twice with 25 mM Tris, 0.2 M glycine, and 20% methanol containing 0.1% $NaDodSO_4$. Proteins were transferred to 0.45-$\mu$m nitrocellulose sheets overnight at 25 V with the above Tris/glycine buffer without $NaDodSO_4$. Following transfer, sheets were incubated for 24 h with phosphate-buffered saline containing 0.05% Tween 20 (PBS/Tween) to prevent nonspecific adsorption. Strips were then incubated for 2 h at room temperature with rabbit antiserum (dilution 1:250 in PBS/Tween) raised against synthetic peptides representing residues 6–21 and 108–121 of angiogenin. After being washed with PBS/Tween, strips were incubated for 2 h at room temperature with 10 $\mu$g of alkaline phosphatase labeled goat anti-rabbit IgG. The strips were then washed with PBS/Tween and treated for 30 min with nitroblue tetrazolium (4.5 mg) plus 2.5 mg of 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidide in 50 mL of 0.1 M barbital buffer. The strips were finally washed with distilled water.

## Results and Discussion

*Transfections and RNA Analysis.* The angiogenin gene that contains a leader sequence was incorporated into the expression vector to allow for secretion of the desired product. Furthermore, a mammalian expression system was employed to ensure proper alignment of disulfide bonds. The two expression vectors employed, pMTAG(+7) and pMTAG(+64), each contained the angiogenin genomic coding sequences (HAGF), the inducible mouse metallothionein promoter (MT-1), and a DHFR selectable marker joined to the SV40 late promoter (Figure 1). The first construct began at +7 and the other at +64 of the angiogenin coding sequence, and both contained ~2 kilobases of 3′ flanking sequence between the TAA and *Eco*RI sites (Kurachi et al., 1985). Following transfection using the standard calcium phosphate precipitation technique, transfectants were selected in media containing increasing concentrations of MTX. Those cells surviving in the highest concentration of MTX, i.e., 1 mM, were expanded and analyzed for angiogenin mRNA and protein production after induction with 80 $\mu$M $ZnSO_4$, 2 $\mu$M $CdSO_4$, or a mixture of the two salts.

Initially, the levels of angiogenin mRNA produced by each of the two expression vector constructs with or without induction by metals were assessed by solution hybridization. For mixed, uncloned cultures selected in 1 mM MTX both types

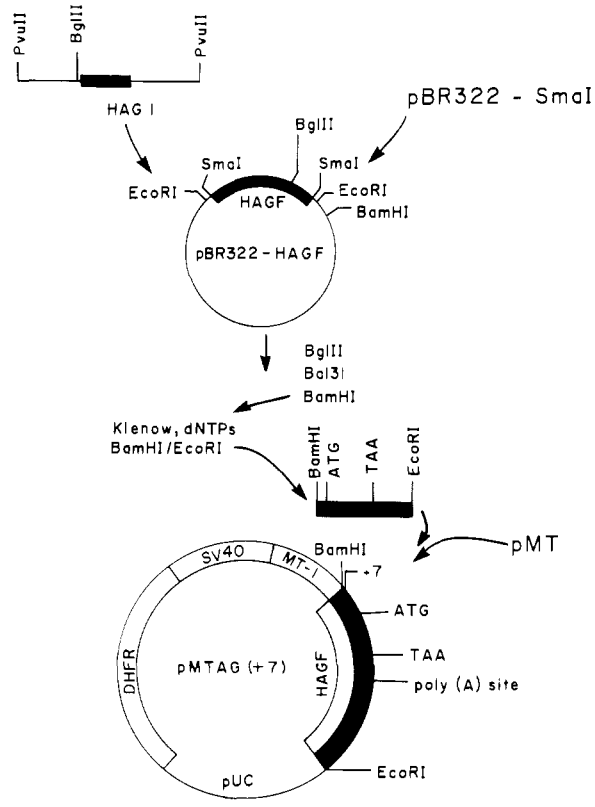


FIGURE 1: Recombinant plasmid pMTAG(+7) for expression of human angiogenin in BHK cells. A genomic *Pvu*II fragment containing the angiogenin coding sequence (HAG1) was cloned into a derivative of pBR322. The resulting plasmid (pBR322–HAGF) was linearized with *Bgl*II and digested with *Bal*31 followed by *Bam*HI. Following blunt-end ligation, the plasmid was amplified in *E. coli* and DNA subjected to restriction analysis. A linearized insert was then generated by *Bam*HI–*Eco*RI cleavage. The insert, starting at +7 from the site of transcription initiation, was then ligated into an expression vector (pMT) to generate the final angiogenin vector. A second construct beginning at +64 from the site of transcription initiation, resulting in increased yields of angiogenin expression, was also constructed (see text).

of transfectants contained ~1280 copies of mRNA/cell in the absence of added metals. When RNA was isolated from cells that had been induced for 8 h in the presence of 2 μM cadmium sulfate or 80 μM zinc sulfate, copy numbers increased 3.2-fold and 2.4-fold, respectively, regardless of the type of construction employed. Several clones of BHK cells were prepared by limiting dilution from cells transfected with pMTAG(+7) or pMTAG(+64) which were selected in 1 mM MTX. Transcription levels for two of these clones, designated BHK(+7) and BHK(+64), reached 15 000–22 000 copies of mRNA/cell when cells were induced with a combination of 2 μM cadmium sulfate and 80 μM zinc sulfate. Induction with either salt alone resulted in diminished levels of transcription (data not shown). Both clones were stable, since this transcription level was maintained in the absence of MTX over many months. Several other clones were produced from cells containing each of the two constructs, but none approached the transcriptional levels reached by BHK(+7) and BHK(+64).

Northern blot analysis of total RNA isolated from either clone showed a hybridizing band with the expected size of ~760 bases (Rybak et al., 1987). The intensity of this band increased proportionally to increases in copy number after induction by metals (data not shown).

*Purification of Recombinant Angiogenin.* Since clones BHK(+7) and BHK(+64) were not distinguishable by RNA analysis, angiogenin was isolated from the medium conditioned

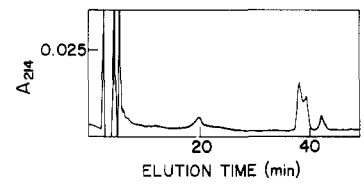


FIGURE 2: Purification of recombinant angiogenin from CM 2 (Fett et al., 1985) by reversed-phase C18 HPLC. The HPLC conditions employed are as described under Experimental Procedures. Angiogenin elutes as two partially resolved peaks at ~39 min.

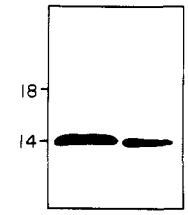


FIGURE 3: $NaDodSO_4$–PAGE in 15% polyacrylamide gels of tumor-derived angiogenin (left lane) and of recombinant material eluting from the C18 column at 39 min (right lane). Positions of molecular weight markers (Bethesda Research Laboratories) are at the left ($\times 10^{-3}$).

Table I: Angiogenic Activity of Recombinant Angiogenin in CAM Assays[a]

| ng | sets of assays | av significance (%) | −SEM | +SEM |
|---|---|---|---|---|
| 100 | 3 | <0.1 | 0.3 | ≪0.1 |
| 50 | 7 | <0.1 | 0.1 | <0.1 |
| 25 | 5 | 0.2 | 0.8 | <0.1 |
| 10 | 6 | 0.1 | 0.3 | 0.1 |
| 5 | 6 | 0.4 | 1.8 | 0.1 |
| 1 | 5 | 1.1 | 2.2 | 0.5 |
| 0.1 | 6 | 5.1 | 13.3 | 1.6 |
| 0.05 | 7 | 11.5 | 16.3 | 4.9 |

[a] Average percent significance and SEM values were calculated as described (Shapiro et al., 1987) with 0.0925 as the probability of obtaining a positive result with a control group of eggs (Fett et al., 1985). Angiogenesis was assessed 68 ± 2 h after sample implantation. A significance level of ≤5% has to be attained for a sample to be considered active.

by cells of each type after induction by the combination of cadmium and zinc salts. As described under Experimental Procedures, a two-step purification employing CM-52 cation-exchange chromatography followed by C18 HPLC was used. A typical HPLC elution profile, depicted in Figure 2, reveals a peak with the same retention time as angiogenin. The expressed protein elutes as two partially resolved peaks, as is the case for angiogenin from both tumor cells (Strydom et al., 1985) and normal plasma (Shapiro et al., 1987). $NaDodSO_4$–PAGE reveals that the recombinant protein has the same molecular weight as angiogenin and is >98% pure (Figure 3). The yield of angiogenin secreted into the medium by metal-induced cells from each clone was estimated by peak area after elution from C18 HPLC as compared to standards prepared from purified plasma-derived angiogenin. These values were ~40 and ~400 μg/L for clones BHK(+7) and BHK(+64), respectively. The higher synthetic capacity observed for clone BHK(+64) most likely reflects the fact that an additional translation initiation site (ATG) beginning at +52, contained in pMTAG(+7), is not present in BHK(+64) cells.

The positioning of an ATG codon may influence the efficiency with which an inserted gene is expressed. Usually, but not always, the initiation of translation occurs at the first 5′ proximal AUG (Kozak, 1980). Thus, our hypothesis is that in cells containing the pMTAG(+7) construct translation

Table II: Amino Acid Composition of Recombinant Angiogenin[a]

| amino acid | residues/mol | amino acid | residues/mol |
|---|---|---|---|
| Asx | 14.83 (15) | Tyr | 4.00 (4) |
| Glx | 10.70 (10) | Val | 4.49 (5) |
| Ser | 8.29 (9) | Met | 0.93 (1) |
| Gly | 8.45 (8) | Ile | 6.88 (7) |
| His | 5.69 (6) | Leu | 6.27 (6) |
| Arg | 12.58 (13) | Phe | 5.00 (5) |
| Thr | 6.75 (7) | Lys | 6.84 (7) |
| Ala | 5.41 (5) | $^1/_2$-Cys[b] | 5.83 (6) |
| Pro | 8.00 (8) | Trp | 1.11 (1) |

[a] Average of seven analyses. Samples were hydrolyzed for 18–24 h at 110 °C with 6 N HCl and analyzed by reversed-phase chromatography after derivatization with phenyl isothiocyanate. Values are presented as residues per mole and integer (in parentheses) from the sequence. [b] Analyzed as cysteic acid after performic acid oxidation. Average of three analyses.

initiates at the first AUG, which occurs at +52. This would result in the synthesis of a peptide out-of-frame with angiogenin. The competing synthesis of this peptide in BHK(+7) cells could explain the lower yields of angiogenin obtained in comparison with BHK(+64) cells, in spite of a similar transcription rate for angiogenin message in both cell types. Interestingly, since some angiogenin is synthesized by BHK(+7) cells, the ribosome can recognize the authentic in vivo translation initiation site at +113 (Kurachi et al., 1985). Kozak (1981) has proposed that purines flanking an AUG codon at positions −3 and +4 from the adenine form a favored consensus sequence for eukaryotic initiation sites. The designated in vivo ATG for angiogenin corresponds to that favored sequence, i.e., GAGATGG at positions +110 through +116 (Kurachi et al., 1985).

Due to this higher yield in secreted, recombinant angiogenin, BHK(+64) cloned cells were propagated in large-scale culture under conditions described above. For large-scale purification Mono S chromatography instead of C18 HPLC was employed (Shapiro et al., 1987). Angiogenin obtained by this procedure was homogeneous as assessed by $NaDodSO_4$–PAGE and was used for further characterization as detailed below. Approximately 400 μg of angiogenin can be purified from 1 L of medium conditioned for 24–36 h. This compares to yields of ~0.5 μg/L obtained from HT-29 colon adenocarcinoma cells by utilizing similar cell culture and purification procedures (Fett et al., 1985). Currently, factories are maintained under the above conditions for ~40 days without deleterious effects on cell viability. Factories are retired when sheeting is observed, presumably due to cell overgrowth.

*Biological and Immunological Characterization.* Table I shows statistical analysis of the CAM data for recombinant angiogenin and indicates that its angiogenic activity is comparable to that of angiogenin derived from either tumor cells (Fett et al., 1985) or normal human plasma (Shapiro et al., 1987) sources. Recombinant angiogenin is specifically rec-

Table III: Compositions of Tryptic Peptides of Recombinant Angiogenin[a]

| | peptide | | | | | |
|---|---|---|---|---|---|---|
| amino acid | 1 | 2 | 3a | 4a + 3b | 5 | 6 |
| Asx | 2.02 (2) | 0.64 | | 2.00 (2) | 0.98 (1) | |
| Glx | 1.11 (1) | 0.31 | | 0.24 | 1.11 (1) | |
| Ser | 0.87 (1) | 0.20 | 0.89 (1) | 2.25 (1) | | |
| Gly | | 0.10 | | 1.21 (1) | | 1.02 (1) |
| His | | | | 0.96 (1) | | |
| Arg | 1.00 (1) | 1.04 (1) | | 1.05 (1) | 1.00 (1) | 1.06 (1) |
| Thr | | | | | | 0.94 (1) |
| Ala | | | | | | 2.00 (2) |
| Pro | | 0.96 (1) | | 1.13 (1) | | |
| Tyr | | | | | | |
| Val | | | | | | |
| Met | | | | | | |
| Ile | | | 1.02 (1) | 2.49 (1) | | |
| Leu | | | | | 0.91 (1) | |
| Phe | | | | | | 0.99 (1) |
| Lys | | | 1.08 (1) | 2.80 (1) | | |
| sequence position[b] | 1–5 | 122–123 | 52–54 | (71–73) + (61–66) | 67–70 | 96–101 |

| | peptide | | | | | |
|---|---|---|---|---|---|---|
| amino acid | 7 | 8 | 9 | 9a | 10 | 11 |
| Asx | 2.06 (1) | 3.22 (3) | 1.73 (2) | | 0.23 | 4.23 (4) |
| Glx | 1.84 (2) | 1.68 | 2.25 (2) | 1.98 (2) | 1.03 (1) | 2.76 (3) |
| Ser | 0.21 | 2.54 | 2.84 (3) | 2.92 (3) | 2.17 (2) | 1.49 (1) |
| Gly | 1.42 (1) | 2.67 (1) | 0.22 | 0.40 | 2.93 (3) | 1.71 (1) |
| His | 1.88 (2) | 1.02 (1) | | | 0.74 (1) | 0.81 (1) |
| Arg | 0.87 (1) | 0.98 (1) | 2.02 (2) | 1.02 (1) | 0.87 (1) | 1.02 (1) |
| Thr | 2.04 (2) | 1.58 (1) | 1.93 (2) | 1.96 (2) | 0.85 (1) | 0.18 |
| Ala | 1.02 (1) | 1.03 | | 0.23 | | 2.15 (2) |
| Pro | 1.05 (1) | 0.87 | 0.73 | 0.58 | 4.23 (4) | 2.13 (1) |
| Tyr | 1.54 (2) | | 1.18 (1) | 1.35 (1) | 1.33 (1) | 0.53 |
| Val | | 0.19 | 1.21 (1) | 1.29 (1) | | 3.42 (4) |
| Met | | | 1.28 (1) | 1.05 (1) | | |
| Ile | 0.73 | 1.89 (2) | 1.02 (1) | 0.98 (1) | | 1.97 (2) |
| Leu | 0.94 (1) | 0.53 | 0.18 | 0.17 | 1.66 (2) | 2.55 (2) |
| Phe | 1.30 (1) | 1.07 (1) | 0.96 (1) | 1.09 (1) | 0.30 | 0.96 (1) |
| Lys | 1.11 (1) | 0.35 (1) | 0.78 (1) | 0.21 (1) | 0.35 (1) | 1.03 (1) |
| sequence position[b] | 6–21 | 41–51 | (22–31) + (74–82) | (25–31) + (74–84) | (34–40) + (83–95) | (55–60) + (102–121) |

[a] Results are reported as residues per mole, with the theoretical compositions in parentheses. Samples were hydrolyzed for 18 h at 110 °C with 6 N HCl, derivatized with phenyl isothiocyanate, and analyzed by reversed-phase chromatography. [b] Based on the sequence of tumor cell derived angiogenin (Strydom et al., 1985).

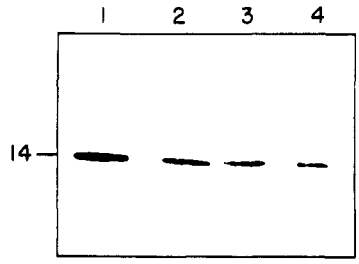


FIGURE 4: Immunological reactivity of plasma-derived and recombinant angiogenin. Purified proteins were electrophoresed on 15% polyacrylamide gels containing SDS followed by transfer to nitrocellulose sheets. Sheets were then reacted with rabbit antibody to synthetic peptides 6–21 (lanes 1 and 2) or 108–121 (lanes 3 and 4), followed by treatment with alkaline phosphatase labeled goat anti-rabbit IgG and the appropriate chromatogenous substrate. Approximately 300 ng of each angiogenin was subjected to NaDodSO$_4$-PAGE: lanes 1 and 3, recombinant angiogenin; lanes 2 and 4, plasma-derived angiogenin.

ognized by two polyclonal antisera directed against two different regions of the angiogenin sequence (Figure 4). The reactivity of equal amounts of recombinant and plasma-derived angiogenin toward the two antisera is essentially the same.

*Enzymatic Activity.* Angiogenin derived from both tumor cells and normal plasma has no significant activity in a variety of standard pancreatic RNase assays using poly(C), poly(U), wheat germ RNA, C>p, U>p, or dinucleotides as substrates (Shapiro et al., 1986, 1987). It does, however, catalyze the limited endonucleolytic cleavage of 28S and 18S rRNAs as determined by agarose gel electrophoresis. Recombinant angiogenin has the same specificity. With wheat germ RNA as substrate there is no detectable formation of acid-soluble material. With 28S and 18S rRNAs as substrates, the degradation pattern is indistinguishable from that observed for both tumor- and plasma-derived angiogenin with major products ranging from 100 to 500 nucleotides. The specific activities measured in this assay are virtually identical, i.e., 0.8 $\mu$M of angiogenin isolated from each of the three sources generates the characteristic cleavage pattern in 30 min at 37 °C.

*Chemical Studies.* Table II shows the amino acid analysis of recombinant angiogenin. Its composition is identical with that of the tumor- and plasma-derived proteins (Shapiro et al., 1987). Automated sequencer degradation of 590 pmol of recombinant angiogenin failed to yield any amino-terminal sequence, indicating a blocked amino terminus, and hydrazinolysis of 515 pmol of protein showed that proline is the carboxyl-terminal amino acid (86% yield).

Tryptic peptides, corresponding to those isolated from the tumor- (Strydom et al., 1985) and plasma-derived (Shapiro et al., 1987) proteins, were isolated. Their amino acid compositions are in agreement with the postulated sequence (Table III). Furthermore, sequence studies on peptides T5–T11 show complete identity to the peptides isolated previously from the natural products.

Hydroxylamine cleavage for an extended period of time [2 days at 25 °C, instead of the 18-h incubation employed earlier (Strydom et al., 1985)] cleaved the two Asn–Gly bonds at positions 61–62 and 109–110; in addition, the Asn–Ser bond at positions 3–4 was also cleaved. Sequencer studies on the core of the hydroxylamine digest revealed two sequences, each identical with the appropriate part of angiogenin and starting at residues 4 and 61, respectively. The small hydroxylamine peptide (the C-terminal peptide consisting of residues 110–123) had a composition and sequence identical with that found previously.

Jointly, the amino acid analyses and sequence studies of the tryptic peptides and hydroxylamine digests account for the expected structure of recombinant angiogenin and establish its identity with the natural product.

The successful large-scale production of biologically active recombinant angiogenin now allows for detailed investigation into the structure–function relationships of this molecule. These will include, but will not be limited to, studies of its pharmacologic role, assessment of therapeutic utility, development of chemical and biological antagonists and/or agonists, and the relationship between enzymatic and angiogenic activities. Changes in structure, i.e., site-directed mutagenesis, will be particularly useful in this regard.

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