第6章　総括

本研究において得られた結論を要約すると、以下のとおりである。

（1）バキュロウィルス-昆虫細胞発現系を用いて、ヒト組織中と同一性を持つ組換えhARを大量に供給するシステムを完成させた。

（2）hARのLys-263は、基質やAR阻害剤との結合に重要な残基であることを、部位特異的変異誘発法によって明らかにした。

（3）hARcDNAを導入し、ほとんど全ての組織にhARmRNAを発現しているトランスジェニックマウスを確立し、糖尿病性合併症類似の病態を作ることに成功した。

なお、本論文第2章の内容は参考論文（1）の内容とほぼ一致し、第3章はBiochemistryに投稿中である（参考論文（2））。

謝辞

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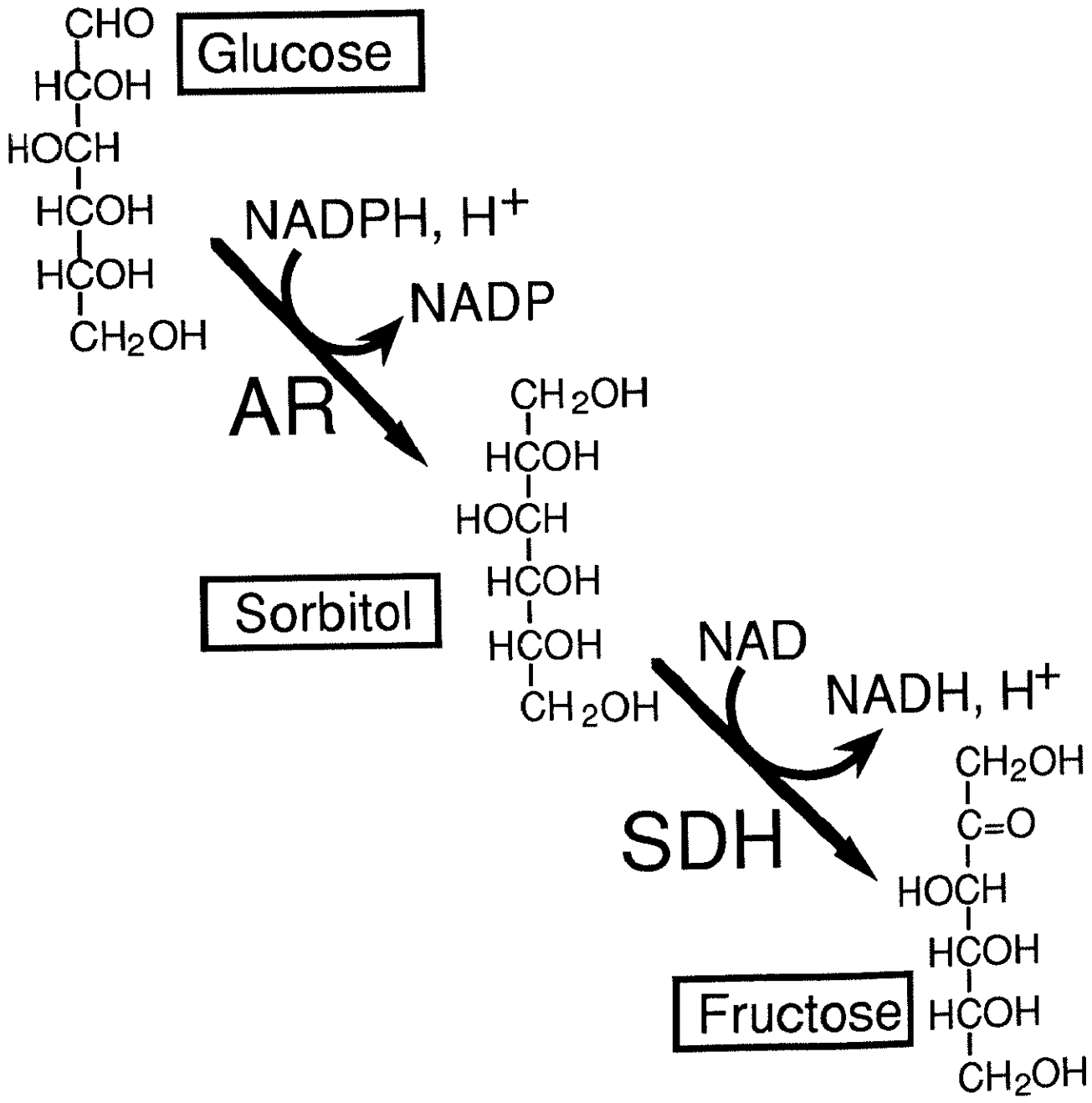
Fig. 1.

POLYOL PATHWAY



AR : Aldose Reductase

SDH : Sorbitol Dehydrogenase

Fig.2.

PATHOGENIC SCHEME OF DIABETIC COMPLICATIONS

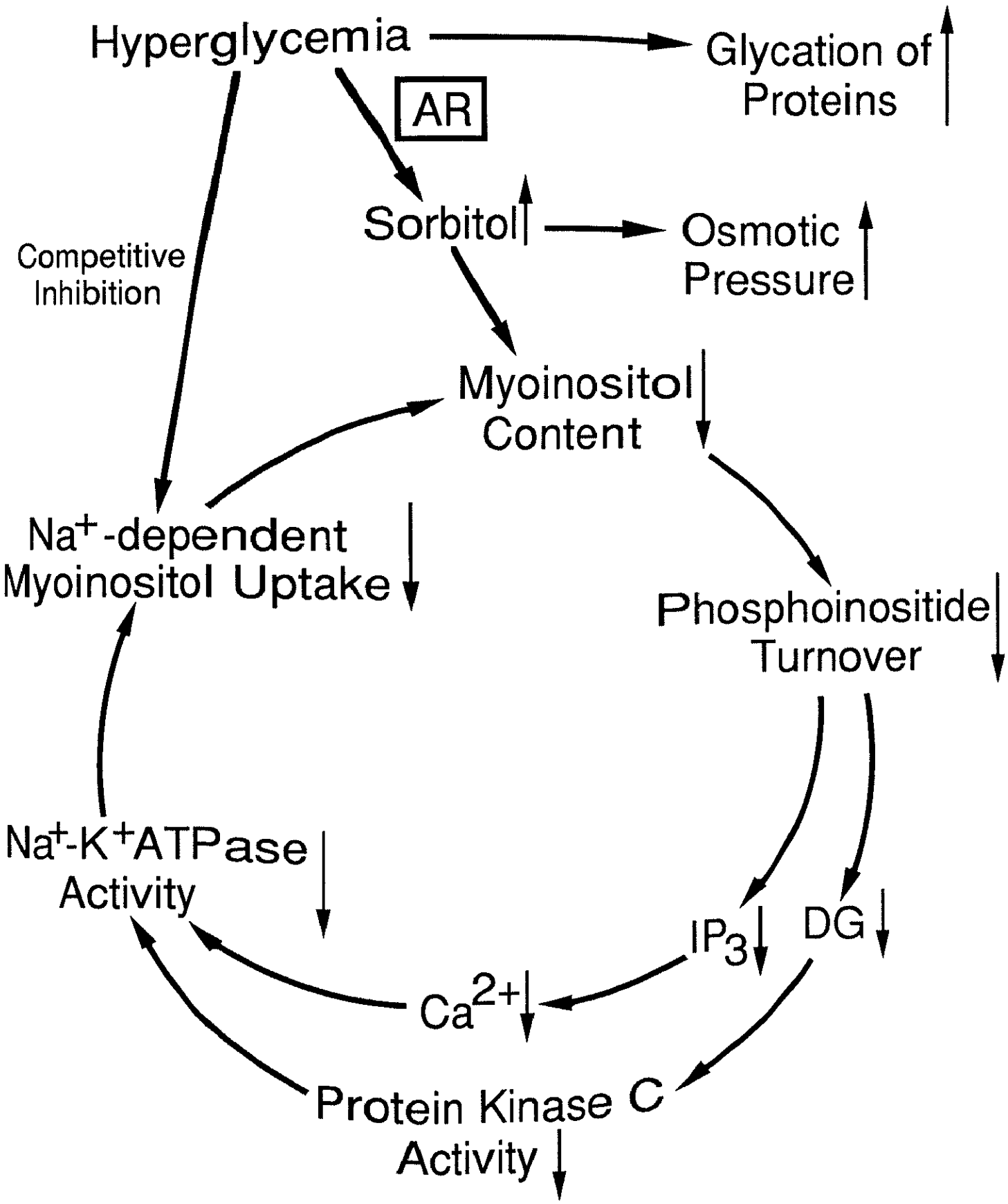


Fig.3.

BACULOVIRUS-INSECT CELL EXPRESSION SYSTEM

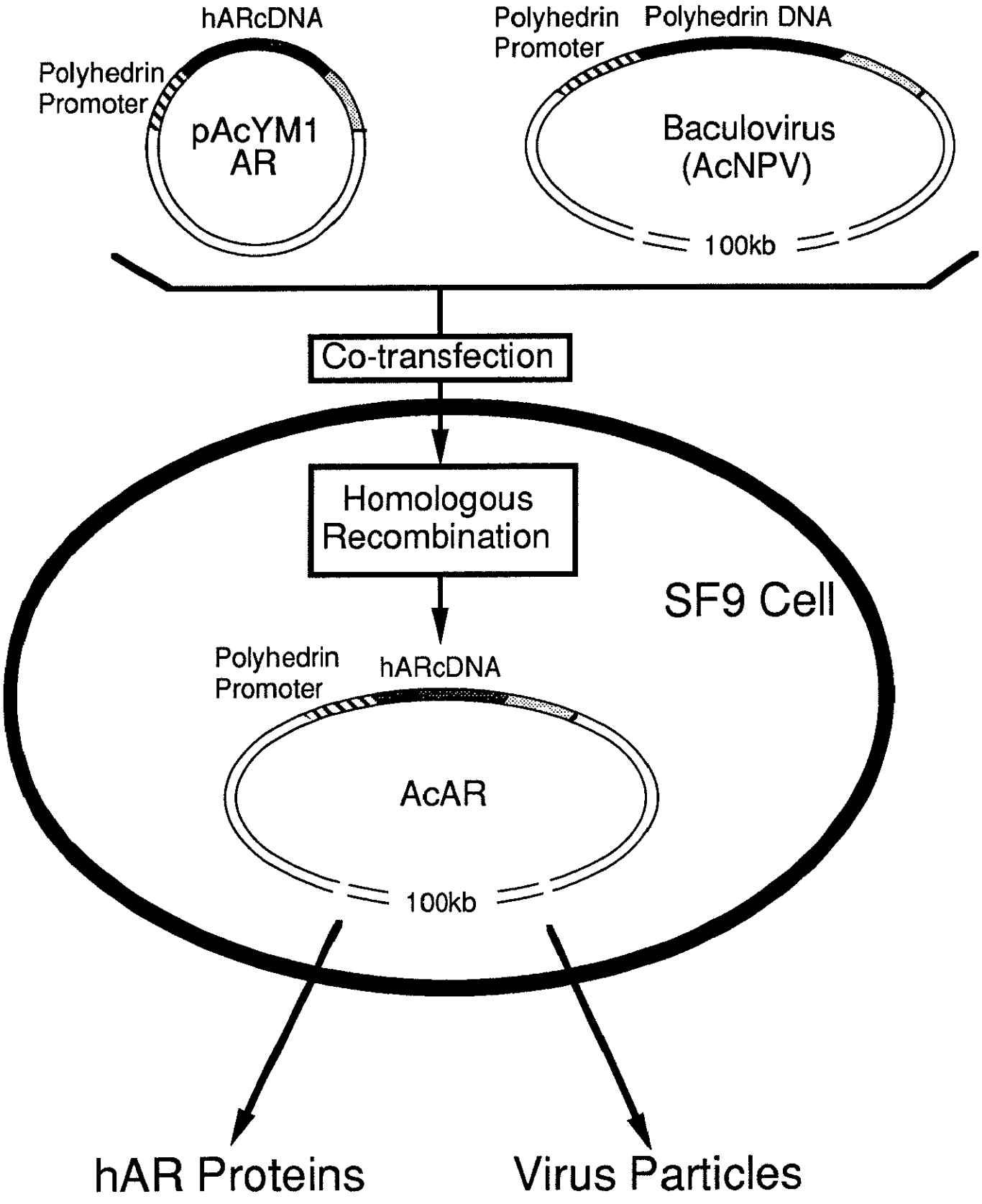


Fig.4.

PROCESSING AND MODIFICATION OF RECOMBINANT hAR

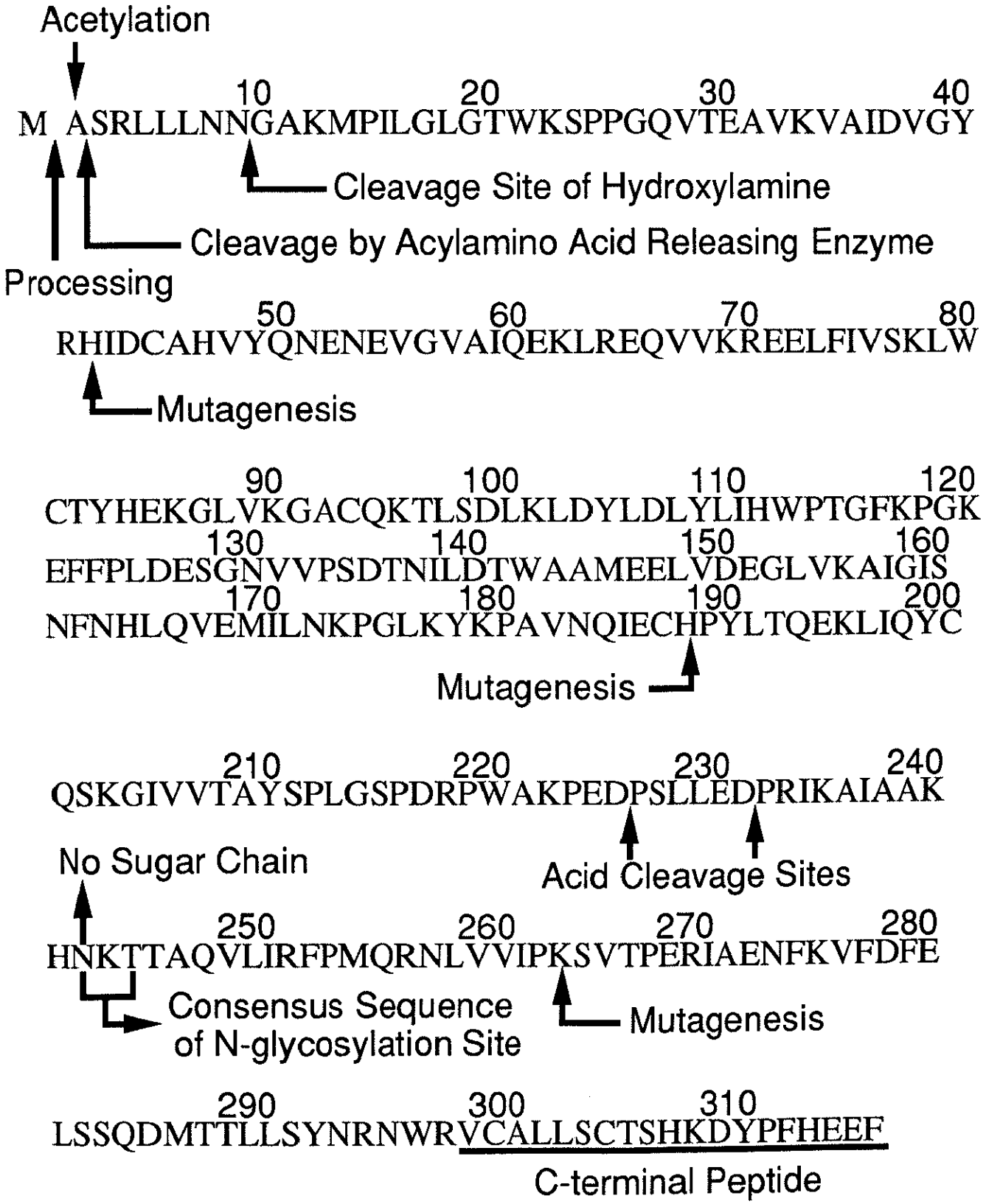
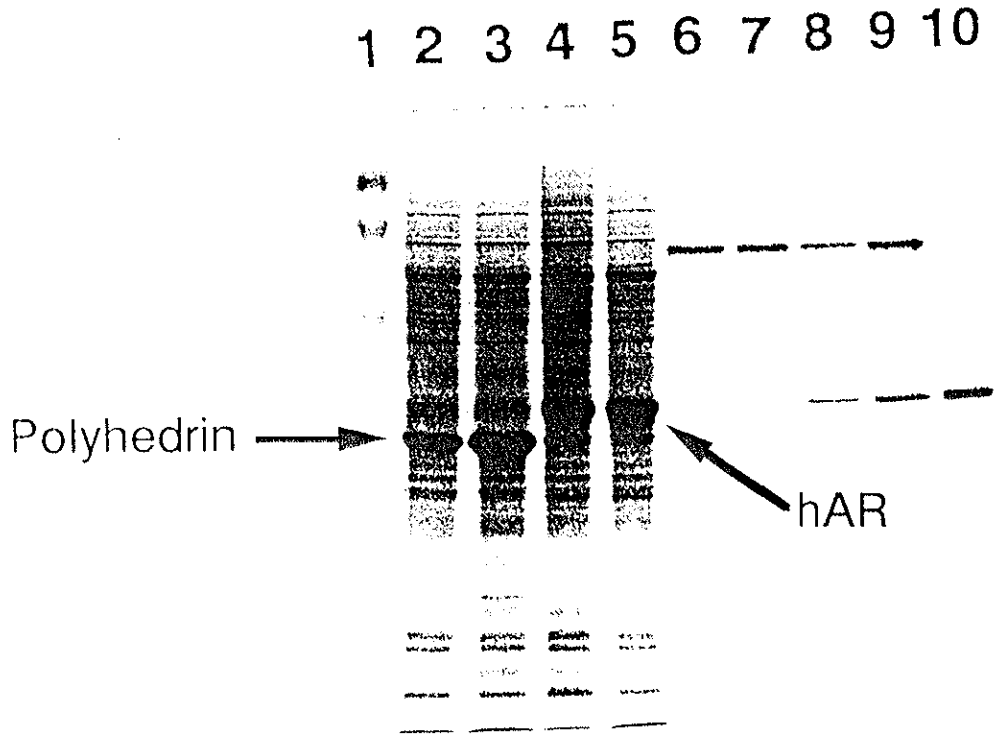


Fig. 5.

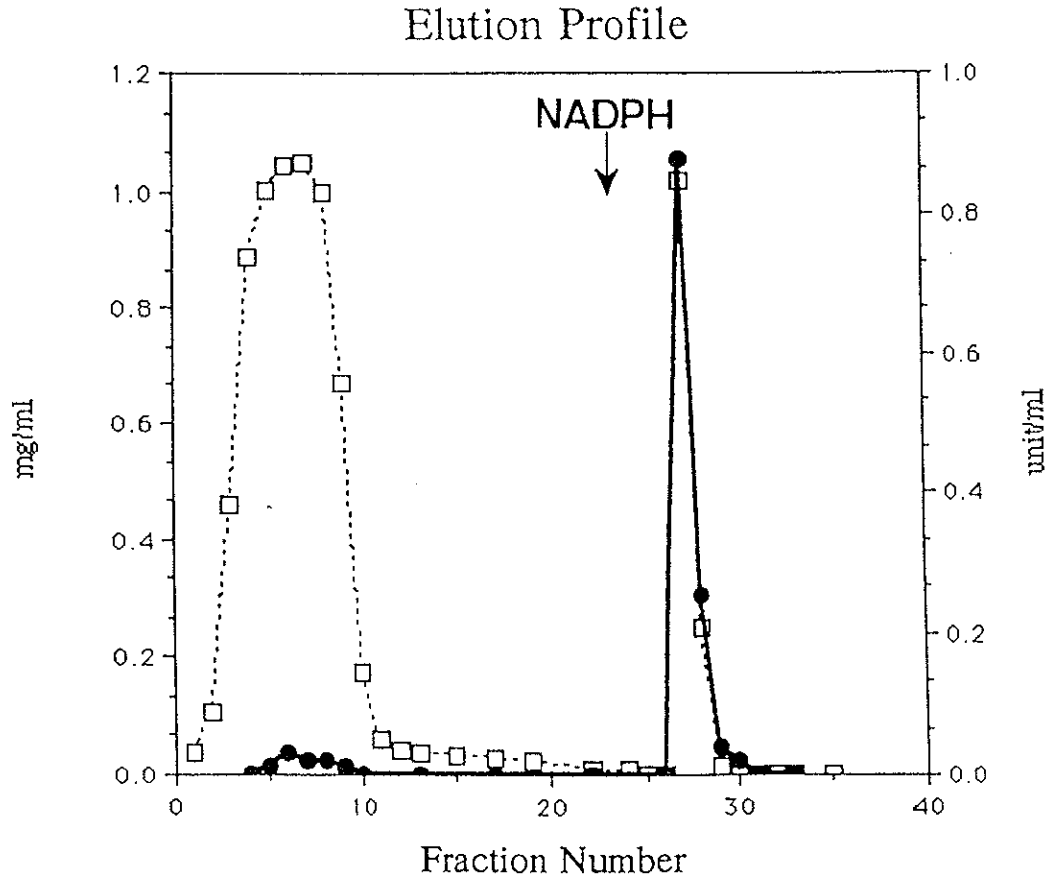
SDS-PAGE DEMONSTRATING EXPRESSION OF hAR



Lane 1, protein markers; lanes 2-5, total cellular proteins of SF9 cells harvested 2 days (lane 2 and 4) or 3 days (lane 3 and 5) after infection with wild type virus AcNPV (lanes 2 and 3) or with recombinant virus AcAR (lanes 4 and 5); lanes 6-9, culture medium of SF9 cells collected 2 days (lanes 6 and 8) or 3 days (lanes 7 and 9) after infection with AcNPV (lanes 6 and 7) or with AcAR (lanes 8 and 9); lane 10, purified hAR from psoas muscle which is a kind gift from Dr. Flynn (64).

Fig. 6.

MATRIX GEL ORANGE A AFFINITY CHROMATOGRAPHY

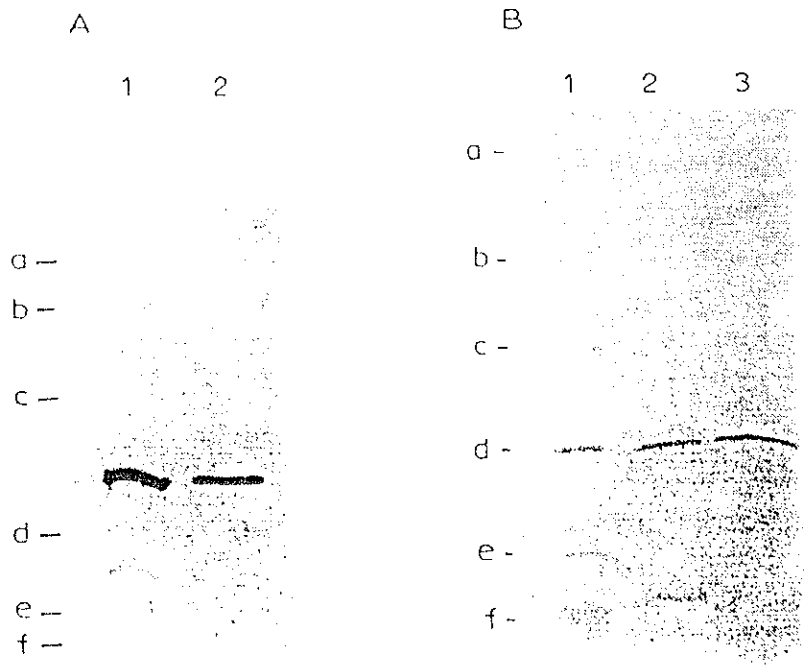


Protein Concentration

AR Activity

Fig. 7.

SDS-PAGE (A) AND ISOELECTRIC FOCUSING (B) OF PURIFIED RECOMBINANT hAR

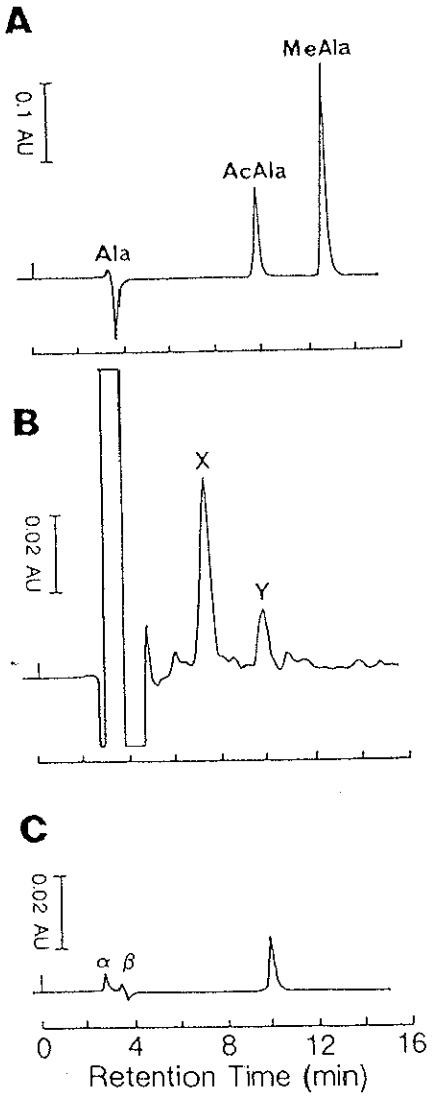


(A) Protein bands of AR purified from human muscle (lane 1), and recombinant hAR eluted from affinity column (lane 2) were detected by silver staining. Marker proteins (a, 94k; b, 67k; c, 43k; d, 30k; e, 20k; f, 14,4k).

(B) The gel was stained with Coomassie R 350. Lane 1, Pharmacia pI Calibration Kit (a, pI=7.35; b, 6.85; c, 6.55; d, 5.85; e, 5.20, f, 4.55; g, 3.50); lane 2, AR purified from human nuscle; lane 3, recombinant hAR.

Fig. 8.

IDENTIFICATION OF N-TERMINAL AMINO ACID OF RECOMBINANT hAR

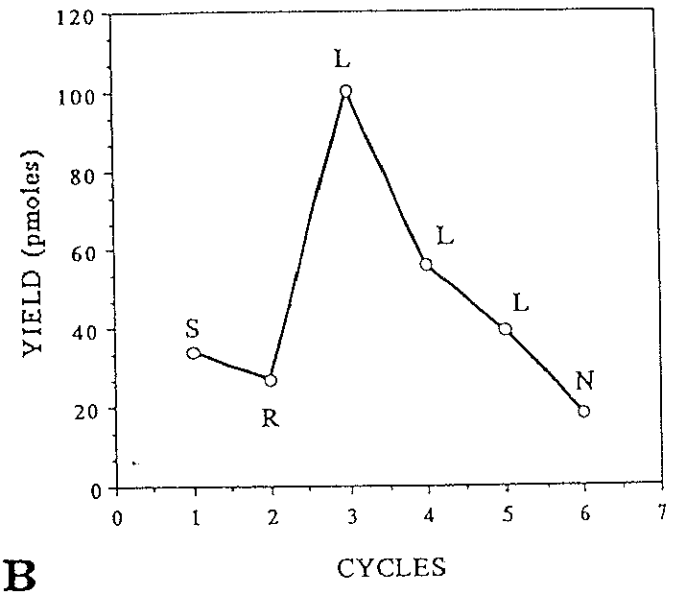


The blocked N-terminal amino acid of recombinant hAR was released by acylamino acid releasing enzyme and analyzed by reverse-phase HPLC using C18 column. Amino acids were separated on isocratic condition in 0.1% TFA at a flow rate of 1ml/min.
(A) Separation of standard amino acids (Ala, alanine; AcAla, acetylalanine; MeAla, metylalanine; 100 nmol, respectively).
(B) Released N-terminal amino acid (peak Y) was identified as acetylalanine. The peak X presumably denotes DTT containing in the reaction mixture.
(C) The eluted peak Y in (B) was incubated at 60 C for 1 h, evaporated to dryness and subjected to the same HPLC analysis. Alanine (peak β) was detected. The peak α presumably represents acetic acid released from acetylalanine by heating with TFA.
Virtical scales indicate absorbance at 210 nm.

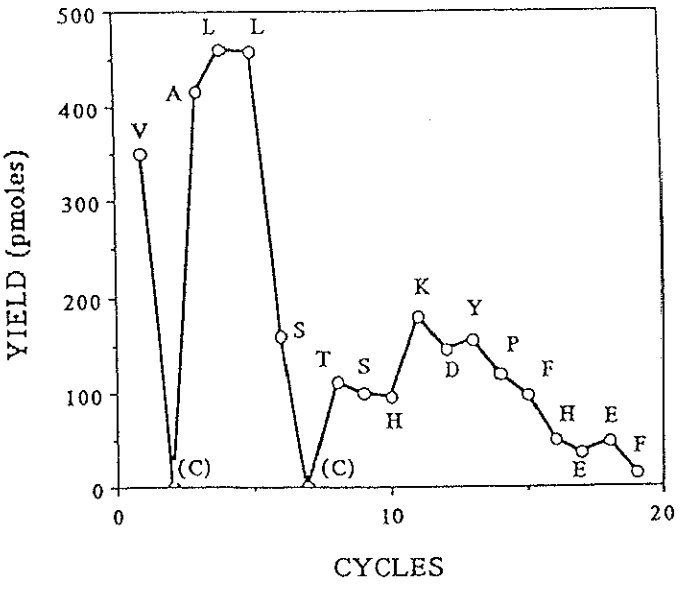
Fig. 9.

AMINO ACID SEQUENCE DETERMINED ON DEBLOKED N- (A) AND C- (B) TERMINAL PEPTIDES OF RECOMBINANT hAR

A



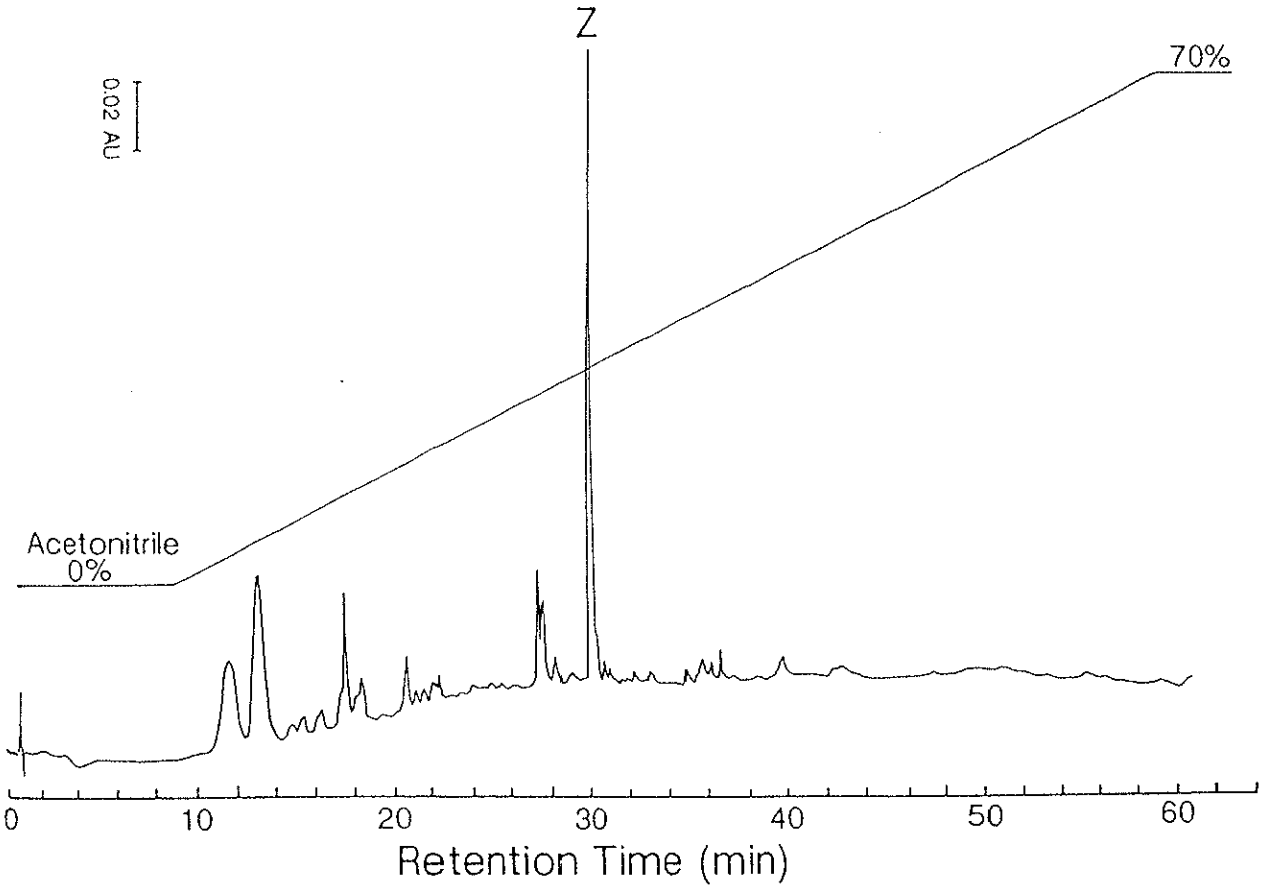
B



Virtical axis indicates the yield of each amino acid after repetitive automated Edman degradation cycle. Because direct sequencing does not allow the identification of cysteine residues, they are designated in parenthesis. Sequence analysis of the C-terminal peptide in the tryptic digest of recombinant enzyme revealed indigestion at Lys(K)-308.

Fig. 10.

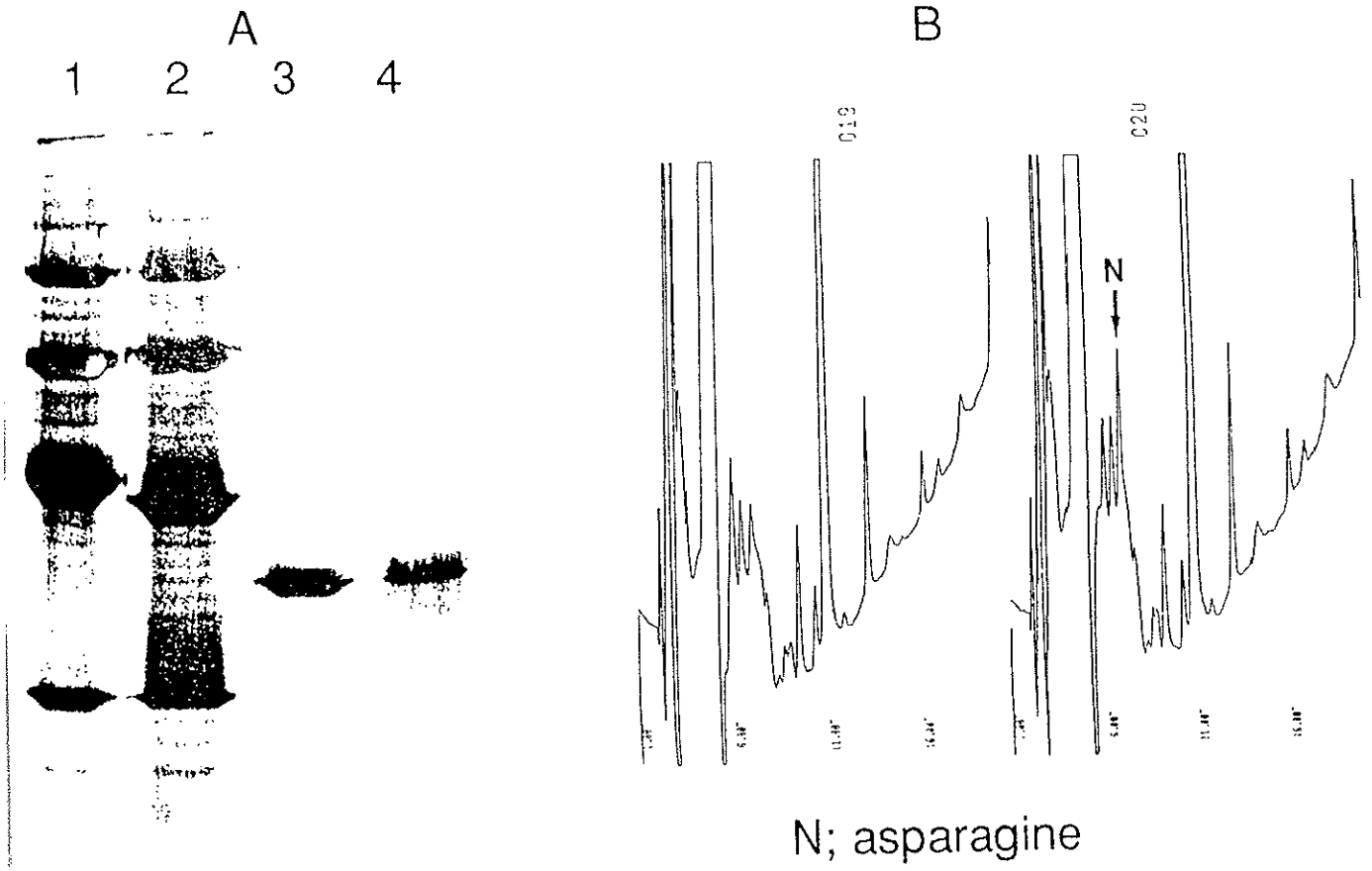
ISOLATION OF C-TERMINAL PEPTIDE OF RECOMBINANT hAR



The C-terminal peptide in the tryptic digest of recombinant hAR was recovered as the flow through fraction of anhydrotrypsin agarose column and purified by reverse-phase HPLC using C18 column with a 0-70% linear gradient of acetonitrile in 0.1% TFA at a flow rate of 1 ml/min. The peak Z was recovered for the following amino acid sequencing. Vertical scale indicates absorbance at 210 nm.

Fig.11.

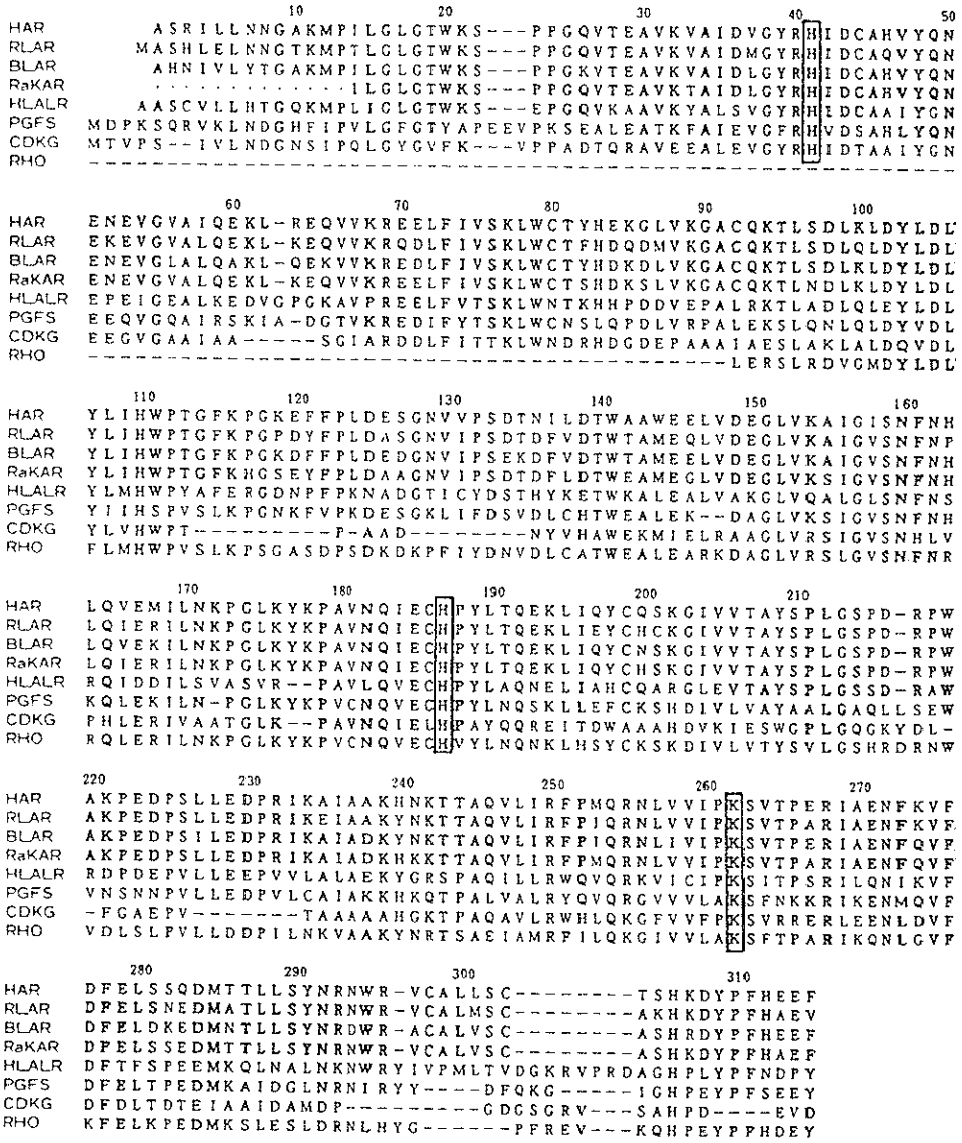
ANALYSIS REGARDING THE POSSIBLE N-GLYCOSYLATION AT Asn-242



(A) SDS-PAGE after N-glycosidase F treatment. Lane 1, marker proteins (94k, phosphorylase b; 67k, bovine serum albumin; 43k, ovalbumin; 20k, carbonic anhydrase); lane 2, marker proteins after N-glycosidase F treatment; lane 3, recombinant hAR; lane 4, recombinant hAR after N-glycosidase F treatment. No significant mobility shift was observed on SDS-PAGE following N-glycosidase F treatment of recombinant hAR. This N-glycosidase F treatment, however, clearly removed sugars from ovalbumin as indicated by a significant shift in mobility toward lower molecular weight on the gel.
(B) Amino acid sequence analysis of the peptide fragment including the possible N-glycosylation site. Asp-Pro bonds of recombinant hAR were cleaved by heating with HCl. The peptide including Asn-242 was separated by SDS-PAGE and transferred onto PVDF membrane with semi-dry blotting method. After Amidoblack 10B staining, the peptide band was cut out and applied to amino acid sequenator. A good yield of the phenylthiohydantoin derivative of Asn-242 is indicative of the lack of sugar moiety attached to the recombinat hAR.

Fig. 12.

HOMOLOGY OF AR SUPERFAMILY



HAR; human AR.
RLAR; rat lens AR (79)
BLAR; bovine lens AR (68)
RaKAR; rabbit kidney AR (80)
HLALR; human liver aldehyde reductase (14)
PGFS; bovine lung prostaglandin F synthase (81)
CDKG; Corynebacterium 2,5-diketo-D-gluconate reductase (82)
RHO; frog lens ρ-crystallin (79)

Fig. 13.

OLIGONUCLEOTIDE-DIRECTED *IN VITRO* MUTAGENESIS SYSTEM

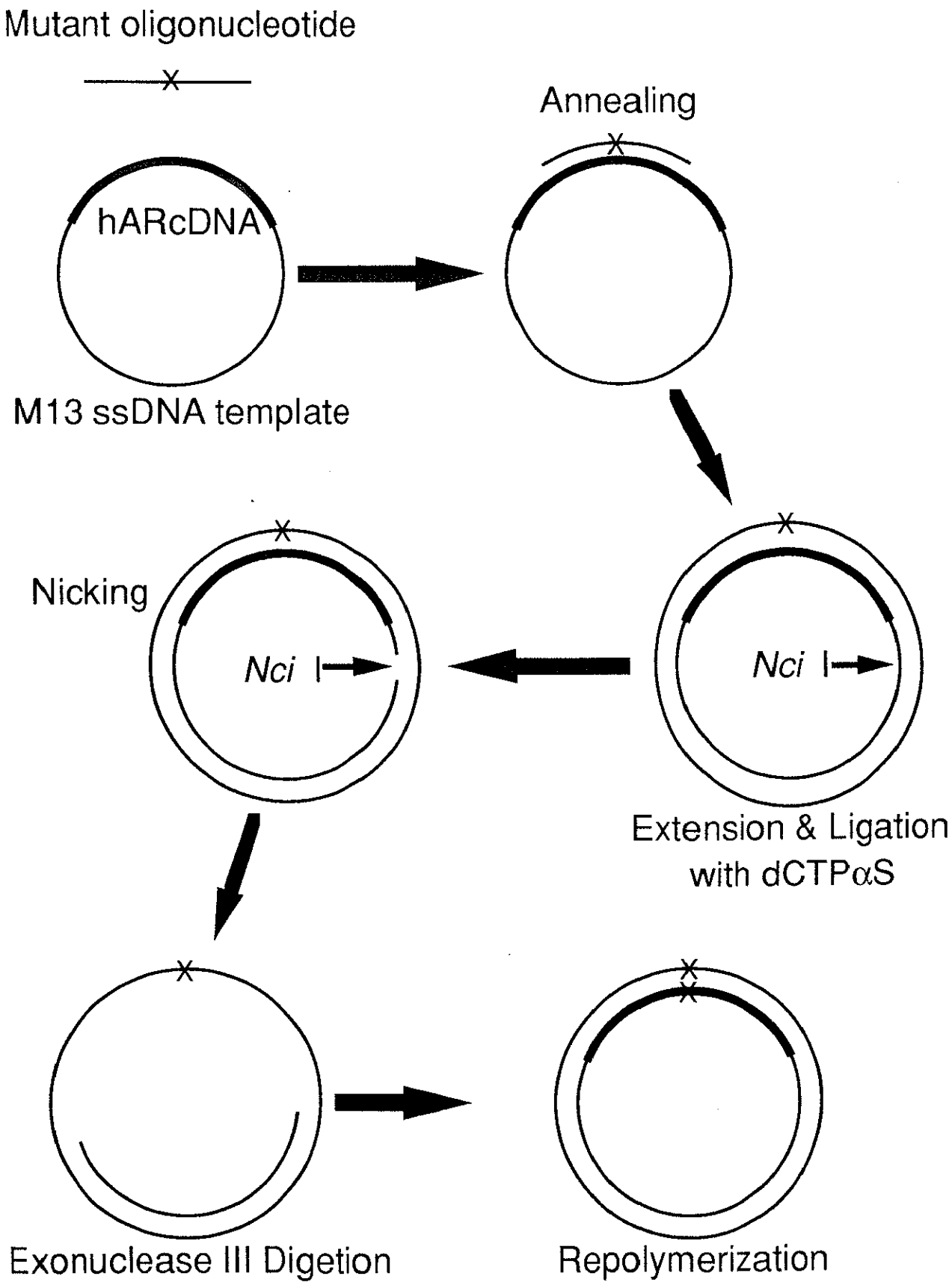
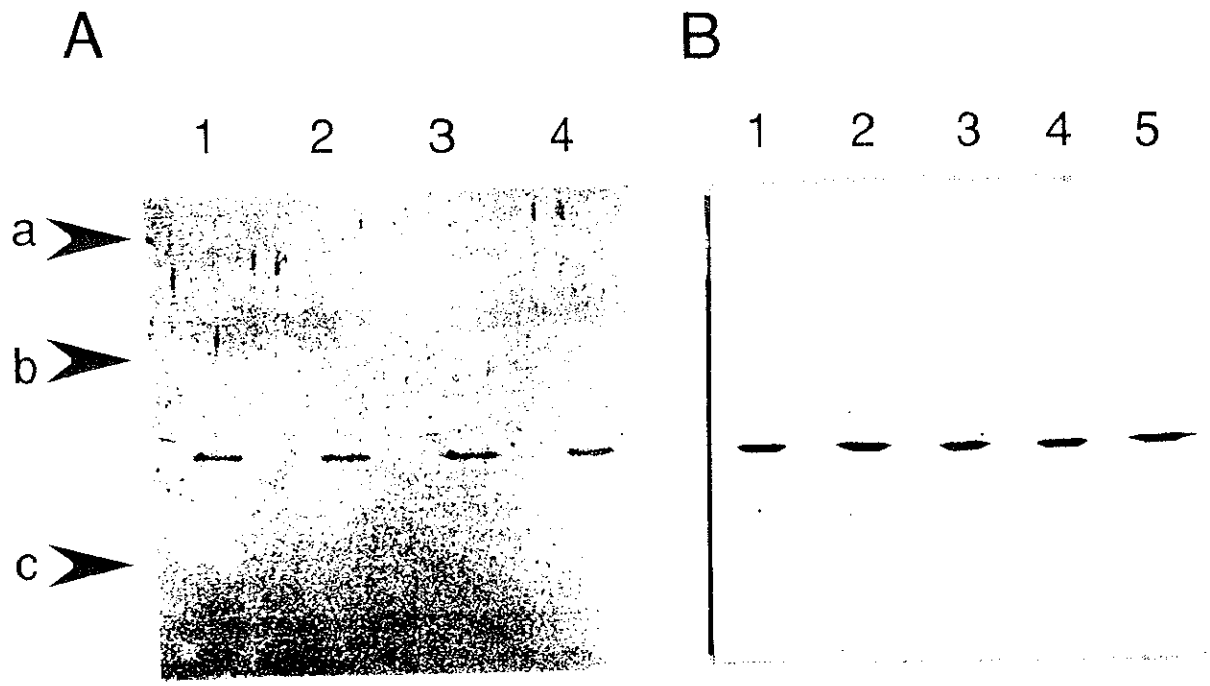


Fig. 14.

SDS-PAGE OF PURIFIED hAR MUTANTS



(A) The wild type AR (lane 1), Met-263 (lane 2), Glu-263 (lane 3), Arg-263 (lane 4) mutants purified by HPLC using hydroxylapatite column were subjected to SDS-PAGE and detected by silver staining. Arrowheads denote the migration of marker proteins (a, 67k; b, 43k; c 30 kd).

(B) Lane 1, the wild type AR; lane 2, Gln-42; lane 3, Tyr-42; lane 4, Gln-188; lane 5, Tyr-188 mutants, respectively.

Fig. 15.

VECTOR FOR hAR TRANSGENIC MOUSE

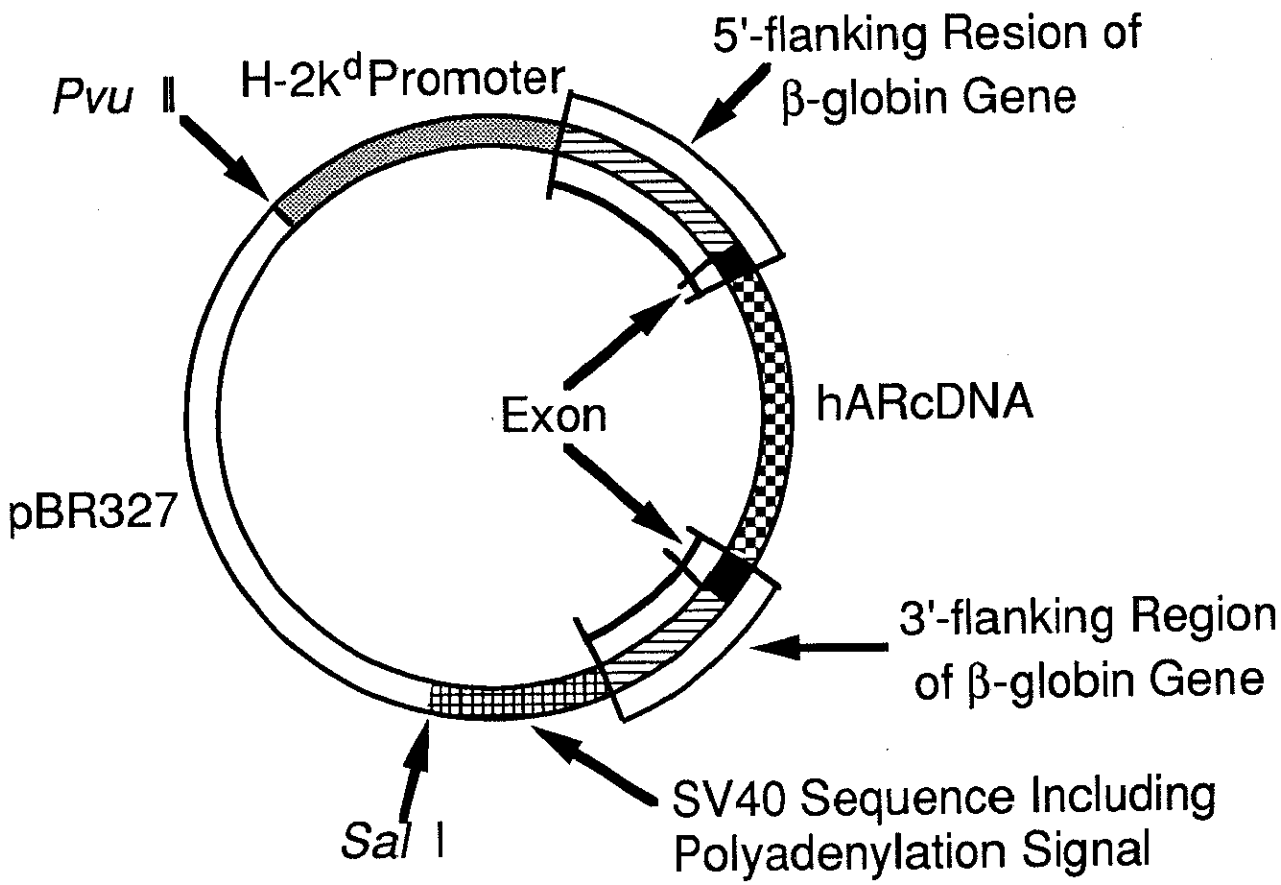


Fig. 16.

PEDIGREE OF hAR TRANSGENIC MICE

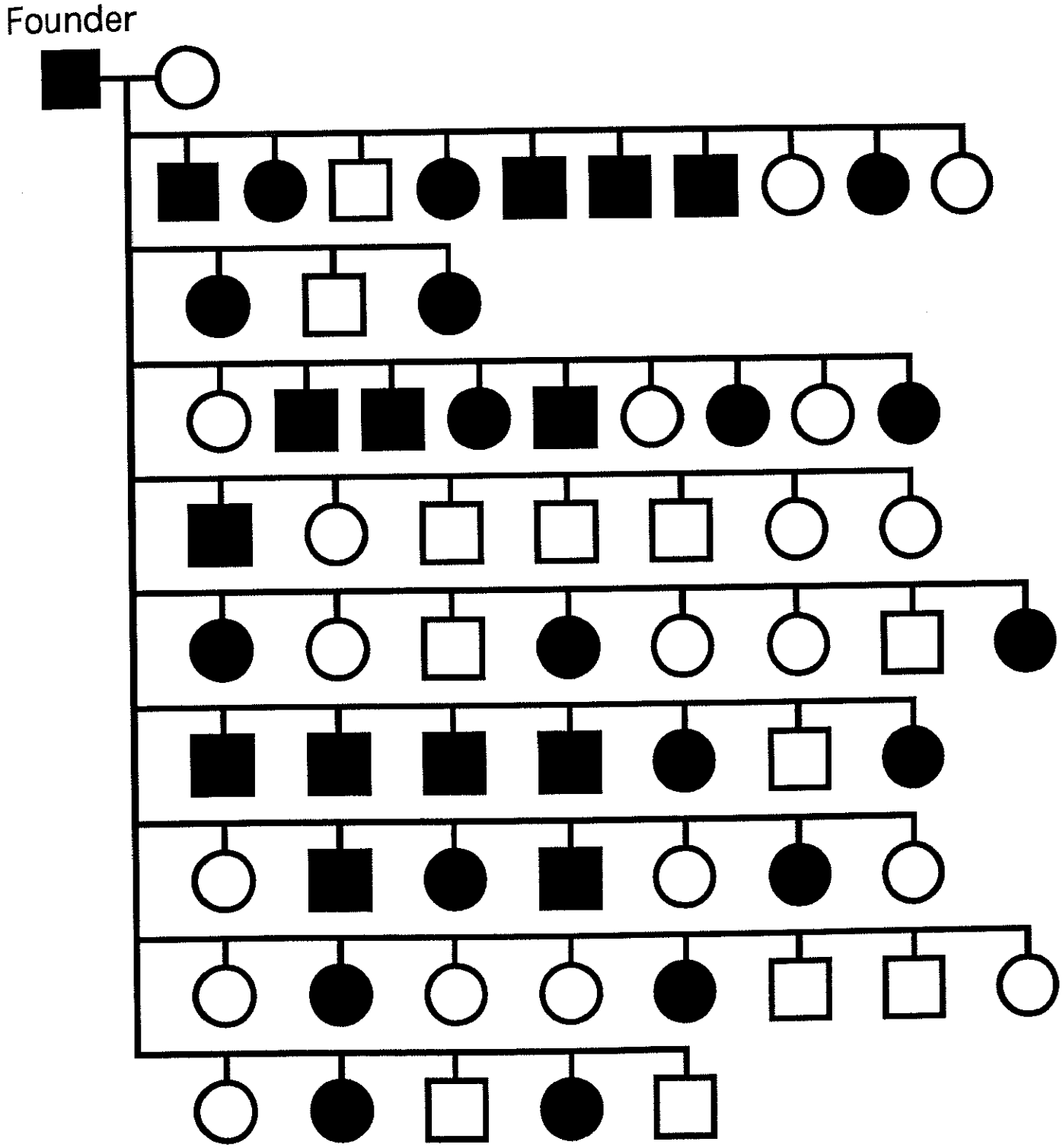
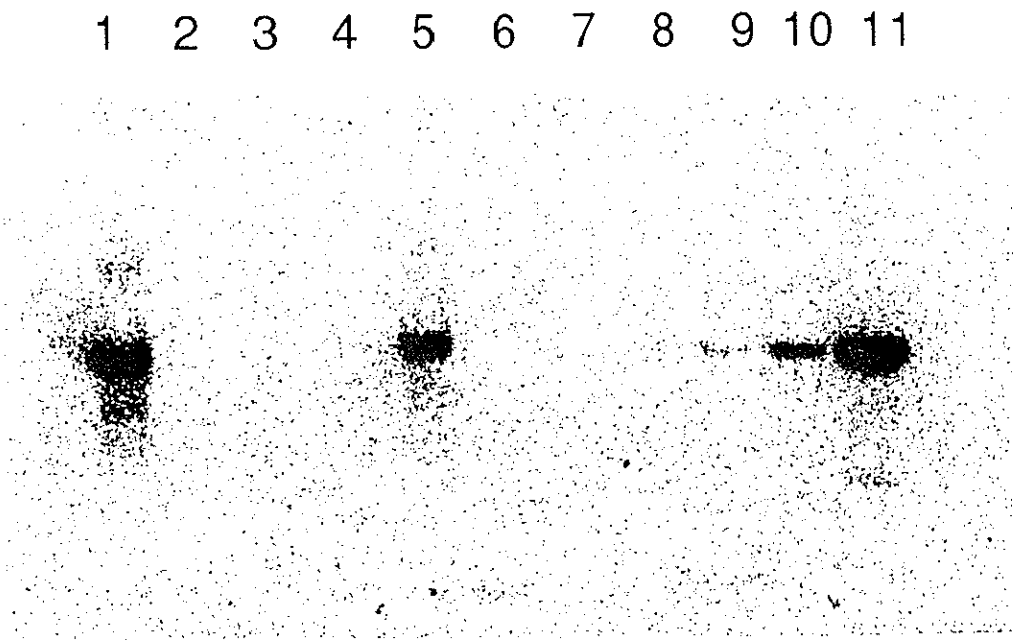


Fig. 17.

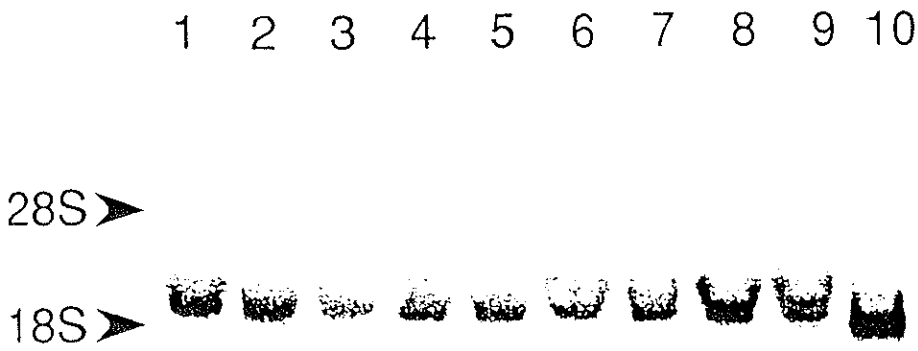
SOUTHERN BLOTTING OF MOUSE TAIL DNA



Two mice (lane 1, 5) have integrated 30-70 copies of hAR, and eight (lane 2, 3, 4, 6, 7, 8) have not. To avoid cross-hybridization to mouse AR gene, the region of hARcDNA between stop codon and polyadenylation signal was amplified by PCR and used as a probe. One, ten, hundred copies of hARcDNA (lane 9, 10, 11, respectively).

Fig. 18.

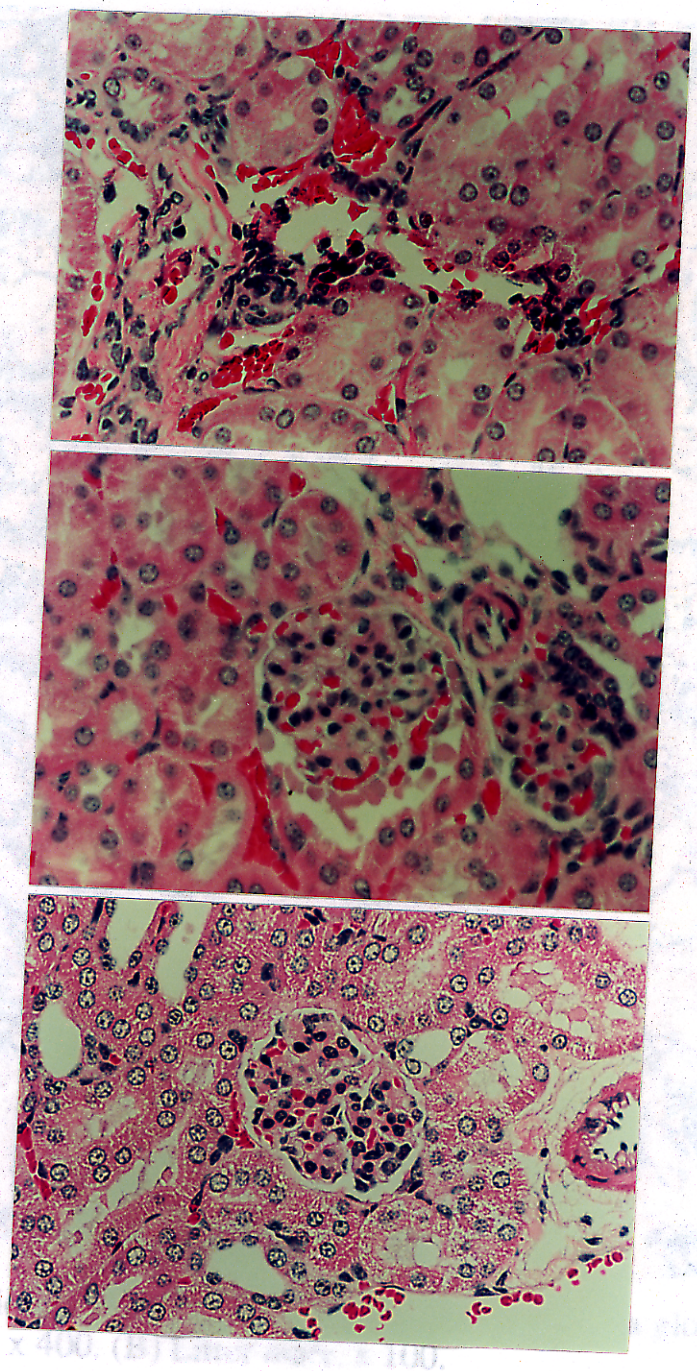
NORTHERN BLOTTING OF hAR TRANSGENIC MOUSE



Total RNA was extracted from each organ of a hAR transgenic mouse by guanidinium thiocyanate / cesium chloride method. The only strand of hARcDNA complementary to mRNA was labeled and used as a probe. The hARmRNA was clearly detected from all organs tested and increased in size than that from HeLa (lane 10), as a positive control. Lane 1, liver; lane 2, thymus; lane 3, muscle; lane 4, heart; lane 5, kidney; lane 6, intestine; lane 7, brain; lane 8, lung; lane 9, spleen.

Fig. 19.

HISTOPATHOLOGICAL CHANGE IN THE KIDNEY OF A hAR TRANSGENIC MOUSE

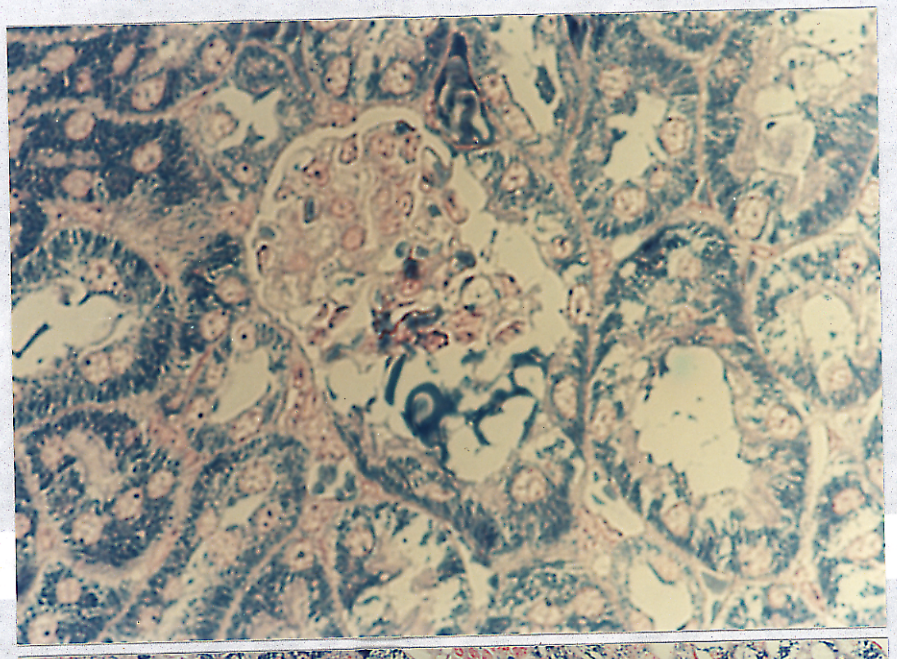


(A) Thrombotic formation in a small artery. HE stain. x 200. (B) Eosinophilic deposits at the periphery of a gromerulus. This manifestation appears to be a diabetic exudative lesion (fibrin cap and capsular drop). HE stain. x 200. (C) Kidney of litter mate. HE stain. x 200.

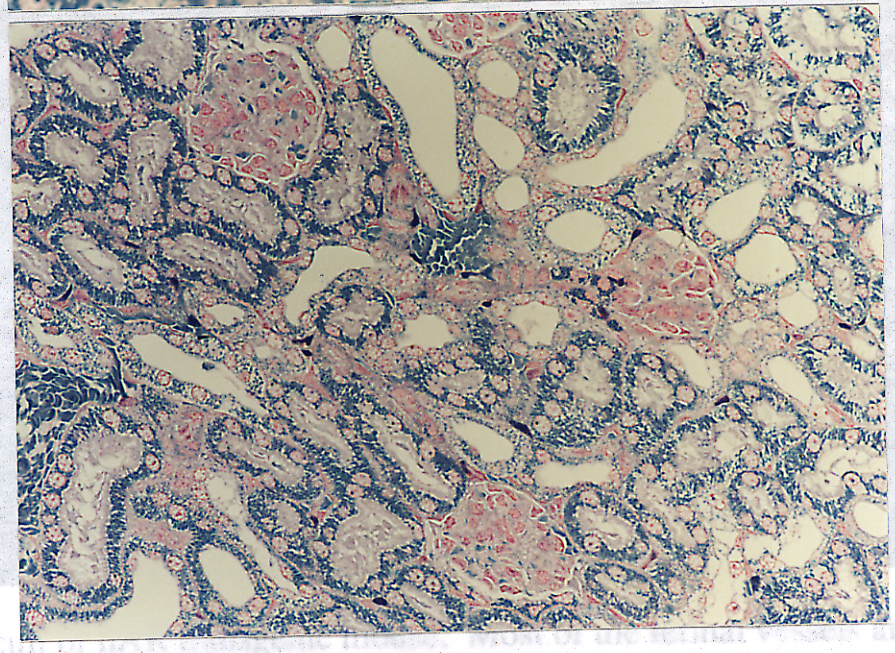
Fig. 20.

PTAH STAINING OF THE DEPOSIT IN THE KIDNEY OF A hAR TRANSGENIC MOUSE

A



B

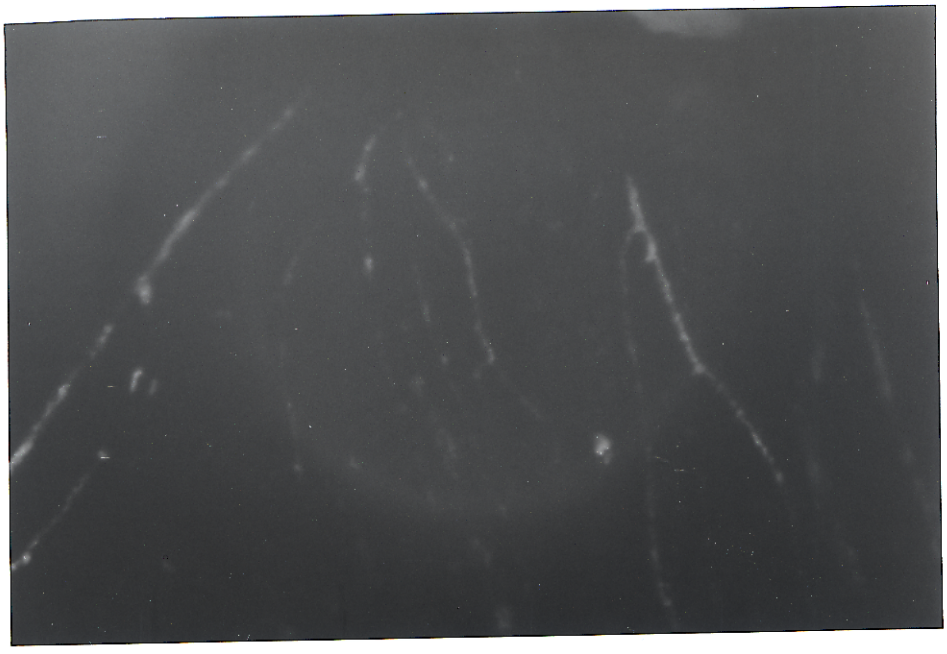


(A) hAR transgenic mouse. The deposit at the periphery of a glomerulus is stained dark blue. x 400. (B) Litter mate. x 100.

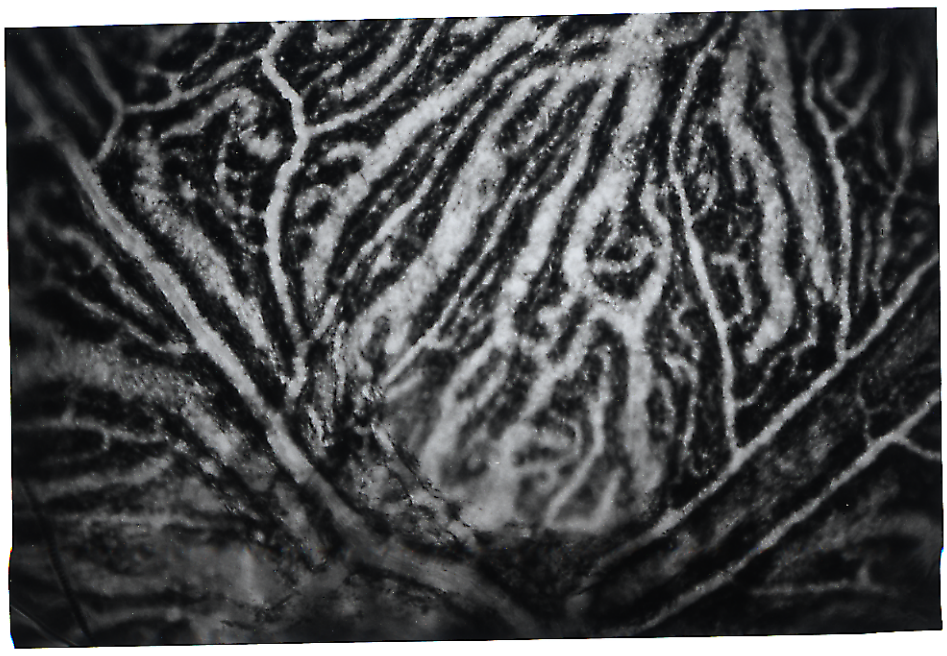
Fig. 21a.

DIABETIC-LIKE RETINOPATHY IN GALACTOSE-FED hAR TRANSGENIC MICE

A



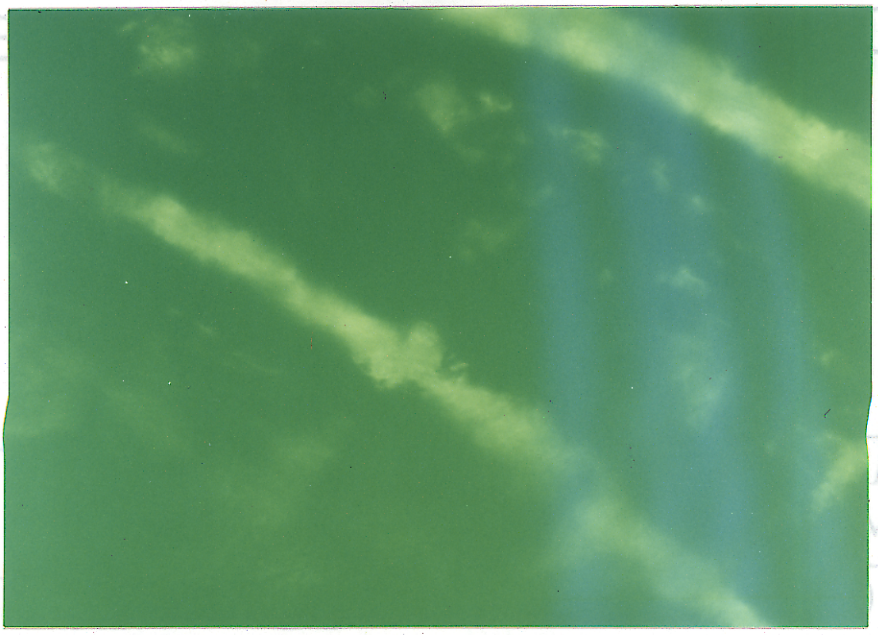
B



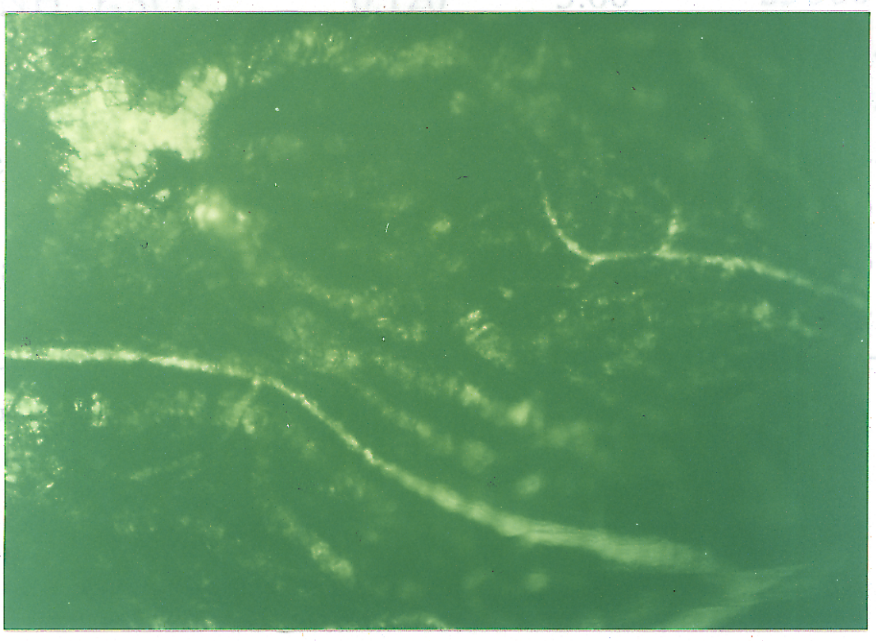
(A) Fundus oculi of hAR transgenic mouse. Most of the retinal vessels are occlusive and have a beaded appearance. Opaque impression of photogram is due to the cataract of the mouse. x 100. (B) Fundus oculi of litter mate. x 100.

Fig. 21b.

C



D



(C) Microaneurysm of the retinal artery. x 400. (D) Macular hemorrhage (left upper). x 200.

Table 1.

SUBSTRATE SPECIFICITY AND THE EFFECT OF SULFATE ION ON KINETIC CONSTANTS OF RECOMBINANT hAR

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\ M^{-1}$)
DL-Glyceraldehyde	0.018	0.673	37400
+0.3 M $(NH_4)_2SO_4$	0.120	3.06	25500
D-Glucose	98.1	0.480	4.89
+0.3 M $(NH_4)_2SO_4$	311	1.17	3.76
D-Galactose	43.3	0.525	12.1
D-Xylose	5.04	0.635	126
D-Glucuronate	3.53	0.418	118
NADPH [a]	0.004	–	–

[a] Assayed with 10 mM DL-glyceraldehyde.

Table 2.

THE EFFECT OF VARIOUS AR INHIBITORS ON RECOMBINANT hAR (RHAR) AS COMPARED WITH AR PURIFIED FROM HUMAN TESTIS (HT), RAT TESTIS (RT), RAT LENS (RL), AND RABBIT LENS (RaL)

Inhibitor	IC_{50} (μM)				
	RHAR	HT [a]	RT [b]	RL [c]	RaL [d]
Sorbinil	0.32	0.55	0.18	0.070	0.75
M79175	0.20	0.44	0.053	0.028	0.079
AL1576	0.014	0.015	0.017	0.024	0.009
Tolrestat	0.018	0.020	0.010	0.011	0.012
Statil	0.011	0.007	0.005	0.016	0.003
Epalrestat	0.26	0.021	0.012	0.010	0.021

a Data from Tanimoto et al. (65).

b Data from Kawasaki et at. (66).

c Data from Sato et al. (67).

d Unbublished data.

Table 3.

OLIGONUCLEOTIDES USED FOR MUTAGENESIS

	Gly-39	Tyr-40	Arg-41	**His-42**	Ile-43	Asp-44	Cys-45
Wild type	GGG	TAC	CGC	CAC	ATC	GAC	TGT
Gln-42		TAC	CGC	CAG	ATC	GAC	TGT
Tyr-42	GG	TAC	CGC	TAC	ATC	GAC	T

	Ile-185	Glu-186	Cys-187	**His-188**	Pro-189	Tyr-190	Leu-191
Wild type	ATT	GAG	TGC	CAC	CCA	TAT	CTC
Gln-188		GAG	TGC	CAG	CCA	TAT	CTC
Tyr-188	TT	GAG	TGC	TAC	CCA	TAT	C

	Val-260	Ile-261	Pro-262	**Lys-263**	Ser-264	Val-265	Thr-266
Wild type	GTG	ATC	CCC	AAG	TCT	GTG	ACA
Met-263	G	ATC	CCC	ATG	TCT	GTG	A
Glu-263	G	ATC	CCC	GAG	TCT	GTG	A
Arg-263	G	ATC	CCC	AGG	TCT	GTG	A

Table 4.

EFFECT OF LYS-263 MUTATION ON KINETIC PARAMETERS OF AR

	DL-glyceraldehyde			NADPH
	Km (μM)	Kcat (s^{-1})	Kcat/Km ($s^{-1}M^{-1}$)	Km (μM)
Wild type	15.5	0.72	465000	4.25
Met-263	211	0.69	3270	35.3
Glu-263	931	0.50	537	15.6
Arg-263	0.83	0.19	229000	21.1

Table 5.

EFFECT OF LYS-263 MUTATION ON INHIBITOR CONSTANTS FOR AR

	Ki (μM)		
	Sorbinil	AL1576	Tolrestat
Wild type	5	0.30	0.067
Met-263	3	0.55	0.043
Glu-263	175	135	0.77
Arg-263	54	1.9	0.32

Table 6.

EFFECT OF HIS-42 OR HIS-188 MUTATION ON INHIBITOR CONSTANTS FOR AR

	Ki (μM)			
	Sorbinil	AL1576	Tolrestat	Statil
Wild type	6.3	0.24	0.037	0.14
Gln-42	17.0	0.28	0.033	0.32
Tyr-42	3.4	0.21	0.018	0.22
Gln-188	11.0	0.32	0.021	0.16
Tyr-188	6.3	0.09	0.007	0.07

Table 7.

EFFECT OF LYS-263 MUTATION ON INHIBITOR CONSTANTS FOR AR

	Ki (μM)		
	Sorbinil	AL1576	Tolrestat
Wild type	5	0.30	0.067
Met-263	3	0.55	0.043
Glu-263	175	135	0.77
Arg-263	54	1.9	0.32