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Blocking of the Initiation-to-Elongation Transition by a Transdominant RNA Polymerase Mutation

MIKHAIL KASHLEV, JOOKYUNG LEE, KATYA ZALENSKAYA, VADIM NIKIFOROV, ALEX GOLDFARB*

RNA polymerase, the principal enzyme of gene expression, possesses structural features conserved in evolution. A substitution of an evolutionarily invariant amino acid (Lys^{1065} → Arg) in the β subunit of *Escherichia coli* RNA polymerase apparently disrupts its catalytic center. The mutant protein inhibited cell growth when expressed from an inducible promoter. The assembled holoenzyme carrying the mutant subunit formed stable promoter complexes that continuously synthesized promoter-specific dinucleotides but that did not enter the elongation step. The mutant polymerase inhibited transcription by blocking the access of the wild-type enzyme to promoters.

DNA-DEPENDENT RNA POLYMERase is a multifunctional enzyme that carries out the principal biochemical steps of gene expression, including specific promoter binding, melting of the DNA double helix, template-directed de novo initiation, processive elongation, and release of RNA at terminators. Although we have a fairly detailed phenomenological understanding of these steps of RNA polymerase functional cycle (*1*), our knowledge of the underlying molecular structures is limited (*2*). These structures, however, must be universal among living organisms, since the general multisubunit architecture of the enzyme (*3–5*) and the primary amino acid sequence of its subunits [(*6*), see Fig. 1A] are remarkably conserved.

We used the molecular genetic approach to study RNA polymerase structure function, focusing on the β subunit of the enzyme from *Escherichia coli*, which is encoded by the *E. coli rpoB* gene (*7*). The β subunit has a homolog in every living organism (Fig. 1A) and has been implicated in each basic step of the RNA polymerase functional cycle (*8*). Our strategy is to engineer in vitro *rpoB* mutations leading to the substitution of evolutionarily conserved amino acids in the hope to selectively inactivate individual basic functions of the enzyme. For example, a mutation might disrupt the polymerase catalytic center so that the enzyme binds to promoters but fails to make RNA. Obviously, such a mutation would be dominantly lethal because the mutant polymerase would in effect become a nonspecific repressor of transcription. For this reason, the mutations are engineered and maintained in a tightly repressed copy of *rpoB*. The second element of our experimental system is the in vitro assembly of a homogeneous preparation of the mutant RNA polymerase from individually overexpressed subunits, which is necessary for a biochemical analysis of the defect. The details of this technology are to be published elsewhere (*9*). Here we describe an *rpoB* mutation that blocks the "escape" of RNA polymerase from the initiation complex, interrupting the transcriptional cycle at an intermediate step.

The sites for mutagenesis were chosen on the basis of affinity cross-linking mapping of amino acids in the vicinity of the RNA polymerase active center (*10*) combined with the homology sequence alignment (Fig. 1A). Two Lys (K) to Arg (R) substitutions, K1065R and K1051R, were constructed in the *rpoB* gene cloned into the pMKA92 expression plasmid (Fig. 1, A and B).

In the genetic system for the analysis of the mutants' phenotype (Fig. 1C), the Rif-sensitive host strain AJ6207 carries amber mutations in its chromosomal *rpoB* and *lacZ* genes and also harbors the Lac repressor–producing plasmid pLacI^{Q}. Such cells are absolutely dependent on the presence of the *supU* suppressor tRNA specified by an F′ episome. Because of this dependence, the host strain never segregates Lac^{-} variants. When the mutant K1051R β subunit was expressed in this host from the pMKA92-derivative plasmid, the cells became rifampicin-resistant. Under these conditions, IPTG-dependent Lac^{-} segregants appeared with the frequency of about 2%, indicating that the plasmid-borne β could complement the chromosomal *rpoB* defect (IPTG, isopropyl-β-D-thiogalactopyranoside). Such a phenotype is identical to the one conferred by the induction of the wild-type β. Thus mutation K1051R did not affect the vital functions of RNA polymerase. In contrast, the AJ6207 cells, as well as other hosts carrying the mutation K1065R encoded in the pMKA92-derivative plasmid, failed to form colonies on IPTG plates altogether, indicating that the cell cannot tolerate the mutate β subunit. Thus we classify the K1051R and K1065R alleles of *rpoB* as silent and dominant negative, respectively. Yet, in spite of its toxicity, the K1065R polypeptide accumulates in substantial amounts when induced with IPTG in liquid culture and is deposited in the insoluble fraction of the cell, as does the wild-type β (*11, 12*).

For the biochemical analysis of the mutants, RNA polymerase holoenzymes containing the K1051R and K1065R β subunits were reconstituted in vitro from individually overexpressed subunits (*13*) and tested in a standard in vitro transcriptional assay with T4 DNA as the template. The specific activity of K1051R polymerase was indistinguishable from that of the intact wild-type polymerase, whereas the K1065R enzyme showed no detectable activity (Fig. 2A). This result correlates with the mutants' phenotypes described above.

We next addressed the question whether the K1065R polymerase would interfere with the functioning of active enzyme in the in vitro system. For this purpose, standard reactions were set up with a limiting amount of T4 DNA template, to which increasing amounts of the mutant polymerase were added together with a fixed amount of normal enzyme. The K1065R enzyme strongly inhibited the normal reaction (Fig. 2B). At a 1:1 ratio of the mutant to the wild-type enzyme, this inhibition was almost fivefold and reproducible. The inhibition was not observed when transcription is performed at DNA excess, suggesting that competition for a DNA target is the basis for the inhibition.

To find out whether the K1065R enzyme forms specific promoter complexes, we used the DNA gel retardation assay (*14, 15*) with a 130-bp DNA fragment carrying the A1 promoter of bacteriophage T7 (*15*). The

M. Kashlev and V. Nikiforov, Institute of Molecular Genetics, U.S.S.R. Academy of Sciences, Moscow 123182, U.S.S.R.
J. Lee, K. Zalenskaya, A. Goldfarb, Department of Microbiology, Columbia University College of Physicians and Surgeons, New York, NY 10032.

*To whom correspondence should be addressed.

wild-type polymerase and K1065R enzyme formed complexes with the promoter fragment that had identical gel mobility (Fig. 3A, lanes 1 and 7). In both cases the complexes remained intact after 2 hours of incubation with poly(dA-dT) (lanes 6 and 12), which sequesters free polymerase instantaneously. However, when the complexes were challenged with the four nucleoside triphosphates (lanes 2 to 5 and 8 to 11), the wild-type and mutant enzymes behaved differently: the normal polymerase complex disappeared, presumably due to elongation and eventual dissociation from DNA, whereas the K1065R enzyme remained bound to the promoter. We conclude that the K1065R enzyme retains the ability to recognize and bind to promoters and to form stable complexes but fails to enter the elongation step of the transcriptional cycle.

To test whether the K1065R polymerase-promoter complex is catalytically active, we determined its ability to synthesize short oligonucleotides in the abortive initiation reaction (*16*). The autoradiogram of Fig. 4 compares the products synthesized on the A1 promoter fragment by the wild-type (lanes 1 and 3) and K1065R (lanes 2 and 4) enzymes. In the presence of the four ribonucleoside triphosphates, the wild-type polymerase (lane 1) yielded three principal products, the dinucleotide pppApU, trinucleotide pppApUpC, and the 65-nucleotide runoff transcript (*17*). Under the same conditions, the K1065R polymerase produced comparatively negligible amounts of all types of transcripts except the dinucleotide pppApU (lane 2). Lanes 3 and 4 of Fig. 4 present a similar experiment performed in transcriptional reactions containing, in addition to the four standard triphosphates, the priming dinucleotide CpA, which corresponds to the positions −1, +1 of the A1 promoter. Because CpA has a much higher affinity to the polymerase initiation site than adenosine triphosphate (ATP), most transcripts synthesized in this reaction are primed with the dinucleotide and thus do not contain phosphates at their 5′ termini. Oligomers of RNA with 5′-hydroxyl group have reduced gel mobility (*18*), as can be readily seen on the autoradiogram. The comparison of the products made under these conditions by the wild-type (lane 3) and K1065R (lane 4) polymerases demonstrates that the mutant enzyme made substantial amounts of the trimer CpApU and relatively much less of the two longer abortive products. No runoff transcript was made. The deficiency displayed by the K1065R enzyme is not specific to T7A1 promoter, since the result of Fig. 4 was reproducible when poly(dA-dT) and the P_R promoter of phage lambda were used as templates. We conclude that, at least qualitatively, the K1065R polymerase is able to catalyze efficiently the formation of the first phosphodiester bond specified by the promoter, whereas its ability to make longer RNA products is substantially reduced.

The properties of the K1065R enzyme resemble the behavior of normal polymerase inactivated by rifampicin (*19*), whose binding site is also located in the β subunit. The models originally suggested for rifampicin action, which are equally applicable to the K1065R defect, explain the failure to elongate RNA by the enhanced loss of oligonucleotides from the ternary transcriptional complex or by the inhibition of transloca-

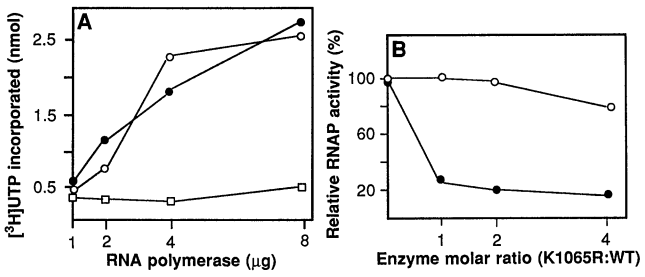

Fig. 2. Analysis of mutant RNA polymerase in the standard in vitro reaction. (**A**) Dependence of the activity on enzyme concentration. Indicated amounts of the wild-type (●) or the reconstituted K1051R (○) and K1065R (□) enzymes were assayed (*22*). (**B**) Effect of K1065R RNA polymerase on transcription performed by the wild-type enzyme. Increasing amounts of K1065R polymerase were added to the transcription reaction performed by 2 μg of the wild-type polymerase on 5.7 μg (○) or 0.6 μg of T4 DNA (●); 100% activity was 2.38 (wild type, DNA excess) and 0.64 (wild type, DNA limit) nmol of UTP incorporated in the standard reaction.

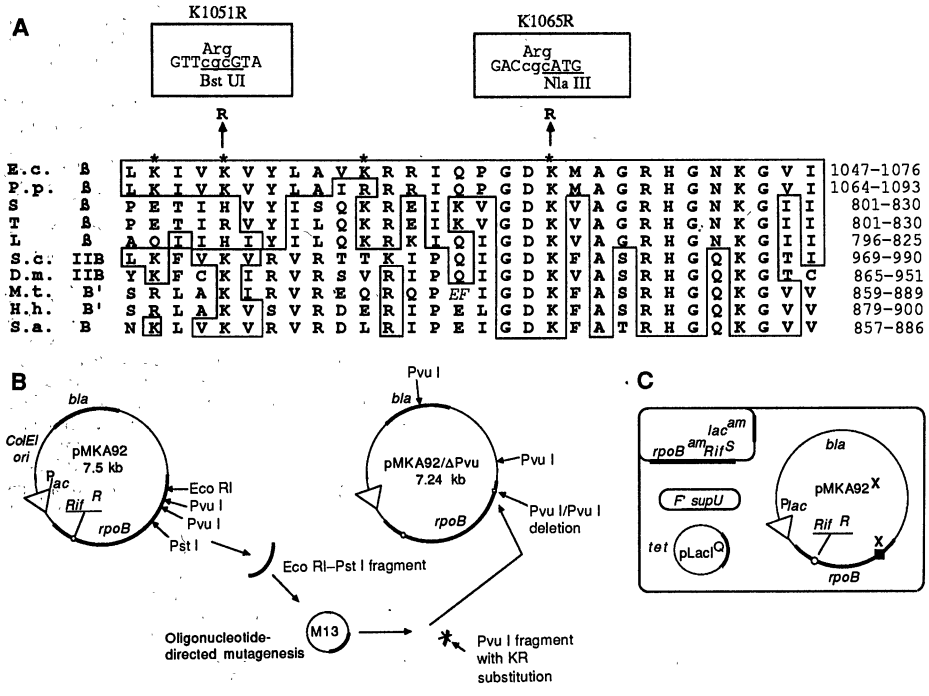

Fig. 1. The *rpoB* mutations K1051R and K1065R. (**A**) Sequence data and homology alignment. Boxed areas present the sequence specifications of the two mutations. The middle line in each box shows the coding DNA sequence at the mutation's site, with substituted nucleotides set in lower case. The arginines specified by the mutations are shown over their codons, and the newly emerged restriction sites are underlined. The amino acid sequences shown (from top to bottom) are from the β subunits of *E. coli* (E.c.), *Pseudomonas putida* (P.p.), chloroplasts of spinach (S), tobacco (T), and liverwort (L); the IIB subunit from *Saccharomyces cerevisiae* (S.c.), *Drosophila melanogaster* (D.m.); and the B(B′) subunit from three species of archaebacteria, *Methanobacterium thermoautotrophicum* (M.t.), *Halobacterium halobium* (H.h.), and *Sulfolobus acidocaldarius* (S.a.) (*6*). The extent of each segment within its respective polypeptide from the amino-terminal to the carboxyl-terminal amino acid is indicated by numbers. Positions of identity to the *E. coli* sequence are outlined. The asterisks over the *E. coli* sequence indicate the four lysines identified as possible alternative labeling sites by substrate cross-linking (*10*). Arrows indicate the two substitutions of lysine (K) to arginine (R). Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (**B**) The genetic engineering scheme based on the *rpoB* expression plasmid pMKA92 (*20*). Bold segments represent genes, and the triangle symbolizes the *lac* promoter. Relevant restriction sites are shown by arrows. (**C**) The genetic system for mutant phenotype analysis (*21*). The engineered mutation is symbolized by X.

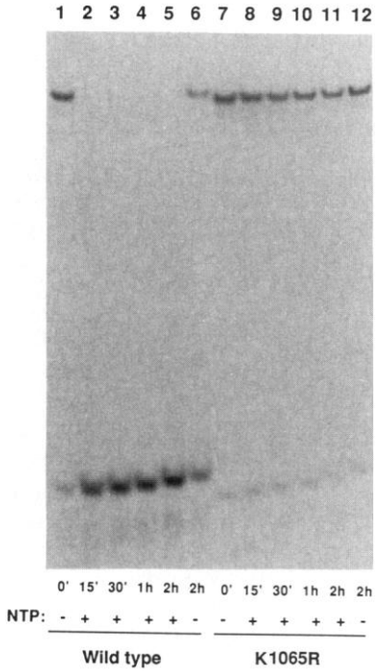

Fig. 3. Gel retardation assay of promoter complexes and their resistance to substrate challenge. The 130-bp promoter DNA fragment that was end-labeled by filling-in reaction at its Bam H1 termini (*15*) was preincubated at 37°C for 15 min with reconstituted RNA polymerase, and then incubated for the indicated time periods with 100 μg/ml of poly(dA-dT) in the absence (lanes 1, 6, 7, and 12) or in the presence (lanes 2 to 5 and 8 to 11) of challenging nucleotides, followed by the gel electrophoresis (*23*).

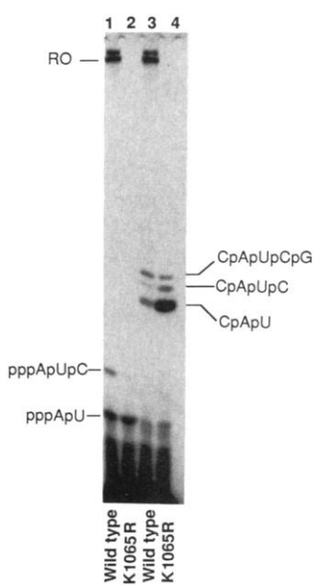

Fig. 4. In vitro activity of the mutant RNA polymerase (*24*). The gel autoradiogram shows the products made by the wild-type (lanes 1 and 3) and K1065R (lanes 2 and 4) enzymes on the 130-bp T7A1 promoter DNA fragment. The transcripts were initiated with ATP (lanes 1 and 2) or CpA (lanes 3 and 4). The oligonucleotide products and the major runoff transcript (RO) are indicated.

tion. Both explanations are consistent with the location of Lys^{1065} side chain in the proximity of the catalytic pocket of the enzyme, as inferred from the cross-linking studies of Grachev and co-workers (*10*).

Basic transcriptional mechanisms can be explored with transdominant RNA polymerase mutations in this experimental system. To study such mutations biochemically, it is necessary to obtain a homogeneous preparation of the mutant polymerase, a difficult task if one were to separate mutant and wild-type molecules from the RNA polymerase pool of the induced cell. Individual overexpression of subunits in combination with the in vitro assembly technology circumvent this problem and make the toxic polymerase amenable to biochemical experimentation.

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8. Individually, the β subunit has no function, but in the assembled enzyme, it at least participates in the forming of structures involved in each stage of transcription as well as in regulatory response. This follows both from genetic evidence [T. Yura and A. Ishihama, *Annu. Rev. Genet.* **13**, 59 (1979); V. Nene and R. E. Glass, *Mol. Gen. Genet.* **194**, 166 (1986); R. E. Glass *et al.*, *ibid.* **203**, 487 (1986); D. J. Jin and C. A. Gross, *J. Mol. Biol.* **202**, 45 (1988)] and from the analysis of RNA polymerase cross-linking to DNA [R. Simpson, *Cell* **18**, 277 (1979)] and substrates [see (*10*)].
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20. The mutations were engineered in the plasmid pMKA92 (*12*), which carries a rifampicin-resistant allele of the gene *rpoB* under the control of the inducible promoter Plac. The Rif-resistant mutation (substitution of Asp^{516} for Tyr) in pMKA92 was sequenced in our laboratory (M. Kashlev, unpublished results). Mutations were engineered by oligonucleotide-directed mutagenesis [T. A. Kunkel, J. D. Roberts, R. A. Zakour, *Methods Enzymol.* **154**, 367 (1977)] in the Eco RI–Pst I *rpoB* fragment subcloned into phage M13mp19. The mutated Pvu I fragment from the M13 insert was recloned into a partial Pvu I digest of pMKA92/ΔPvu carrying the frameshifting deletion of the Pvu I segment. Successful clones were identified by the replacement of the frameshift marker resulting in the reappearance of the full-size inducible β polypeptide, which was visualized by SDS gel electrophoresis. The generation of mutations was confirmed by sequencing the DNA of M13 inserts in both orientations and by the emergence of the new restriction site at the mutation's location (Fig. 1A).
21. The host strain for phenotype analysis, AJ6207 *rpoB(am) metB argG lacZ(am) rpsL(Str^R)/F′(F14SupU)* [V. Nene and R. E. Glass, *Mol. Gen. Genet.* **188**, 399 (1982)] was provided by R. Glass. The $pLacI^Q$ plasmid, which constitutively expresses the lac repressor, was constructed by inserting the 1.5-kb Eco RI fragment with the $lacI^Q$ coding sequence from pMC9 [T. V. Huynh, R. A. Young, R. W. Davis, in *DNA Cloning, A Practical Approach*, D. M. Glover, Ed. (IRL Press, Washington, DC, 1985), pp. 49–78] into the Eco RI site of the *cat* gene of pACYC184.
22. RNA polymerase was assayed by measuring the incorporation of [^{3}H]UTP (uridine 5′-triphosphate) into acid-insoluble form with phage T4 DNA as template under conditions of core saturation with the σ factor. The standard assay conditions and purification of native RNAP from *E. coli* MRE-600 were as described by S. Malik and A. Goldfarb [*J. Biol. Chem.* **259**, 13292 (1984)].
23. The reaction conditions were 40 m*M* tris-HCl, *p*H

7.9, 10 m*M* $MgCl_2$, 50 m*M* KCl, 7 m*M* β-mercaptoethanol, 20% (v/v) glycerol, 0.2 pmol of the 130-bp T7A1 promoter DNA fragment, and 1 pmol of RNA polymerase. The final volume of each reaction was 20 μl, and the final concentration of challenging nucleotides were 0.25 m*M* each of ATP, GTP, CTP, and UTP. Gel electrophoresis and autoradiography were according to (*14*).

24. The transcription reactions were performed in a 15-μl volume containing 0.1 pmol of the 130-bp T7A1 promoter DNA fragment and 0.5 pmol of RNA polymerase in 40 m*M* tris-HCl, *p*H 7.9, 10 m*M* $MgCl_2$, 7 m*M* β-mercaptoethanol, 100 m*M* KCl, 5% (v/v) glycerol, 0.1 m*M* each of [α-^{32}P]UTP (5 to 10 Ci/mmol), CTP, and GTP. The ATP concentration was 0.25 m*M* in lanes 1 and 2 and 0.1 m*M* in lanes 3 and 4. The concentration of CpA in lanes 3 and 4 was 0.25 m*M*. Incubation was for 15 min at 37°C. The reactions were stopped and the samples were directly applied to a polyacrylamide–8*M* urea gel (20% acrylamide and 3% *N*,*N*′-methylene-bisacrylamide), which were run and autoradiographed as described [S. Malik, M. Dimitrov, A. Goldfarb, *J. Mol. Biol.* **185**, 83 (1985)]. The oligonucleotide products were identified by using different nucleoside triphosphates as the source of α-^{32}P label.

25. We thank H. Heumann for the promoter DNA, R. Glass for a strain, and M. Chamberlin and B. Krummel for comments. M.K. and V.N. thank Soros Foundation for support during their stay in New York. Supported by the PHS grant GM30717 and the ACS grant MV-285 to A.G.

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Localization of PDGF-B Protein in Macrophages in All Phases of Atherogenesis

RUSSELL ROSS, JUNICHI MASUDA, ELAINE W. RAINES, ALLEN M. GOWN, SHOGO KATSUDA, MASAKIYO SASAHARA, L. TREVOR MALDEN, HIDEYUKI MASUKO, HIROSHI SATO

Lesions of atherosclerosis occur in the innermost layer of the artery wall and consist primarily of proliferated smooth muscle cells surrounded by large amounts of connective tissue, numerous lipid-laden macrophages, and varying numbers of lymphocytes. Growth-regulatory molecules may be involved in intimal accumulation and proliferation of smooth muscle cells responsible for the occlusive lesions of atherosclerosis. Platelet-derived growth factor (PDGF) B-chain protein was found within macrophages in all stages of lesion development in both human and nonhuman primate atherosclerosis. Thus macrophages may play a critical role in the disease by providing PDGF, a potent chemotactic and growth-stimulatory molecule, to the intimal smooth muscle cells.

ATHEROSCLEROSIS IS A DISEASE OF large- and medium-sized arteries and is characterized by focal thickening of the inner portion of the artery wall in association with fatty deposits (*1*). This results in the clinical sequelae of myocardial infarction, cerebral infarction, gangrene of the extremities, and loss of function. Fatty deposits, although common in Western society, may be missing in populations where other risk factors such as hypertension, cigarette smoking, and diabetes are associated with increased incidence of the disease (*2–6*). The common underlying events responsible for the formation of lesions are the intimal proliferation of smooth muscle cells, formation of new connective tissue matrix by these cells, and the possible accumulation of lipid. Smooth muscle cells appear in the lesions of experimentally induced atherosclerosis concomitant with, or following the appearance of, monocyte-derived macrophages and T cells (*7–10*). The smooth muscle cells may be derived from existing intimal cells or from cells that migrate in from the underlying media. As the smooth muscle cells proliferate within the intima, the lesion increases in size and forms a fibrous cap that overlies a deeper region of macrophages, T cells, and debris, culminating in a large connective tissue–rich lesion that contains varying amounts of lipid. Platelet-derived growth factor (PDGF) (a homodimer of PDGF-A or PDGF-B chains or an AB heterodimer) may be one of the principal growth-regulatory molecules responsible for the migration of medial smooth muscle cells into the intima and for the proliferation of existing collections of intimal smooth muscle cells. The source of PDGF is not well defined; PDGF may be secreted by platelets, monocyte-derived macrophages, the overlying endothelial cells, or the smooth muscle cells themselves (*6, 11, 12*).

By Northern (RNA) analysis, we examined the expression of growth-regulatory molecules and their receptor genes in advanced lesions of atherosclerosis induced in nonhuman primates that had been made hypercholesterolemic by ingesting a diet rich in cholesterol for 1 year. In comparison with the dissected normal aortas from control animals, transcripts for several growth factors and their receptors were increased in the advanced lesions, whereas others remained unchanged (Fig. 1): PDGF-B, c-*fms* (the colony-stimulating factor type 1 receptor), the β subunit of the PDGF receptor, interleukin-1 (IL-1), and transforming growth factor β1 (TGF-β1) were increased, whereas PDGF-A and β-actin were not. These observations are consistent with the Northern analysis of human carotid endarterectomy specimens in which increased lev-

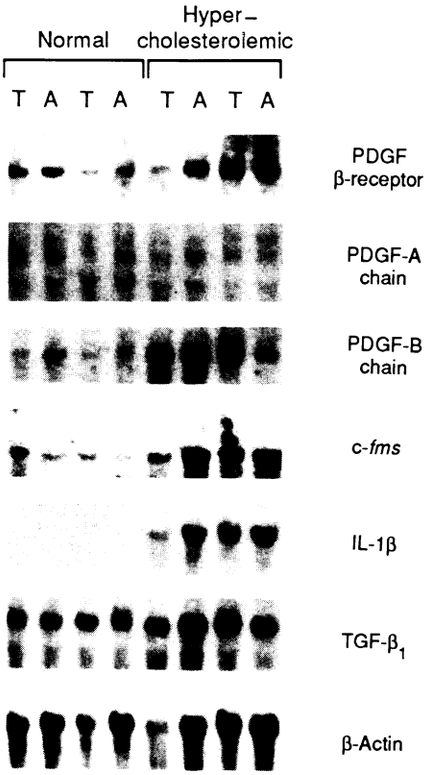

Fig. 1. Comparison by Northern (RNA) analysis of arterial segments of nonhuman primates maintained on a hypercholesterolemic diet (600 to 800 mg/dl) for 1 year with control normocholesterolemic animals. Two pigtail monkeys (*Macaca nemestrina*) between 3 and 5 years of age were maintained on a hypercholesterolemic diet as described (*8*) and killed after 1 year on the diet. An additional two monkeys received normal monkey diet and served as controls. Segments (1 cm) of thoracic (T) or abdominal (A) aorta were removed from each animal and dissected as described for preparation of tissues for immunocytochemistry (*10*) and snap frozen for isolation and analysis of total RNA (*27*). The same blot was sequentially rehybridized with the indicated cDNA probes or cRNA probe. β-actin hybridization was used as a reference for relative mRNA load per lane. Message sizes were 5.7 kb for the PDGF receptor (β subunit); 2.8, 2.3, and 1.9 kb for the PDGF-A chain; 3.7 kb for the PDGF-B chain; 4.3 kb for c-*fms*; 1.8 kb for IL-1β; 2.5 kb for TGF-β1; and 2.1 kb for β-actin.

R. Ross, E. W. Raines, A. M. Gown, S. Katsuda, M. Sasahara, L. T. Malden, Department of Pathology, University of Washington, Seattle, WA 98195.
J. Masuda, National Cardiovascular Center, Research Institute, Osaka, Japan.
H. Masuko and H. Sato, Mochida Pharmaceutical Co., Ltd., Tokyo, Japan.