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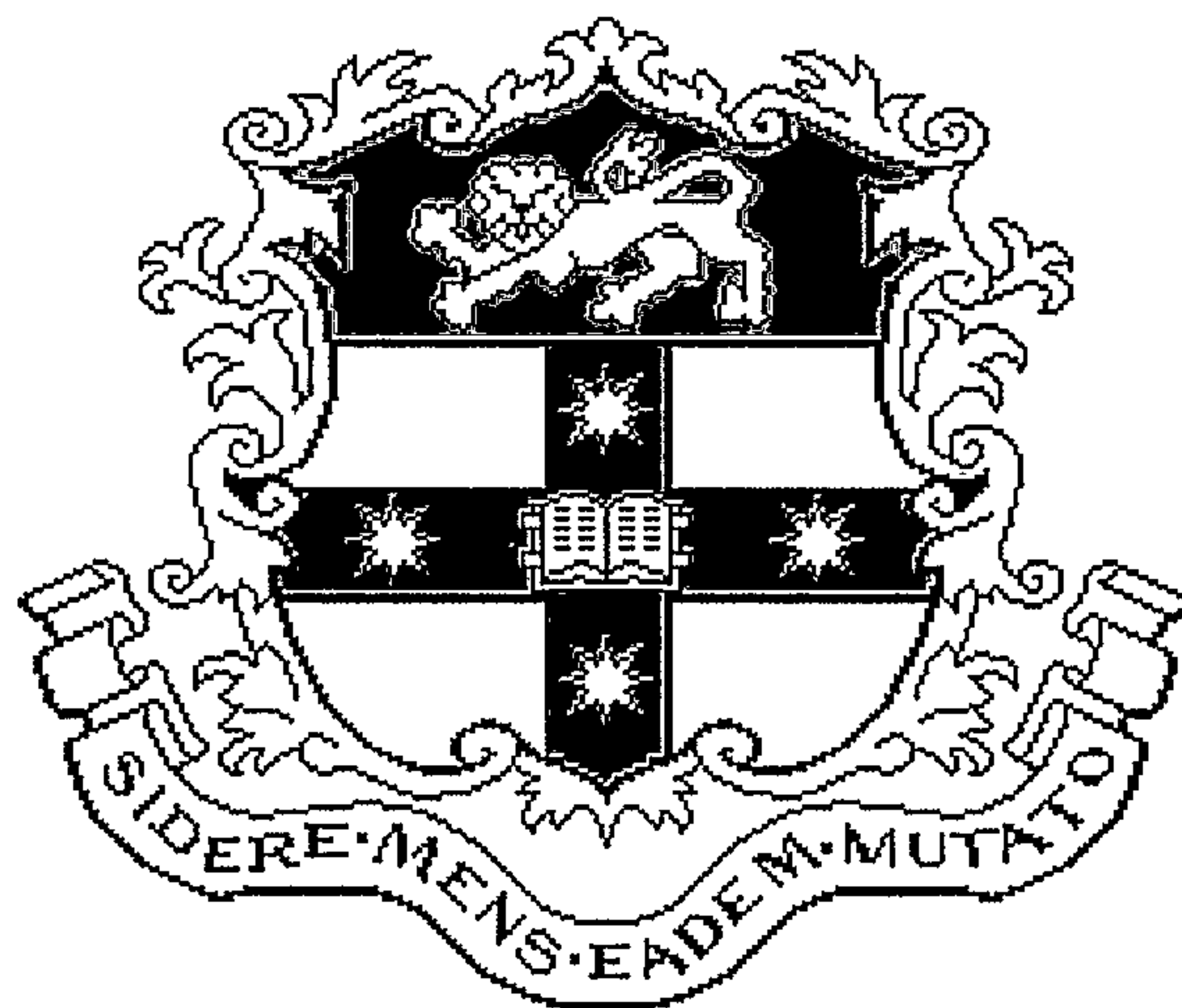
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Cellular and molecular control of root resorption

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A thesis submitted in fulfilment of the requirements for the Degree of Master in

Dental Science in the Faculty of Dentistry, University of Sydney

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Australia

Dedication

I dedicate this thesis to my family.

Thank you always.

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Gratitude goes to the postgraduate MDSc students who have had to endure me during my stressful times.

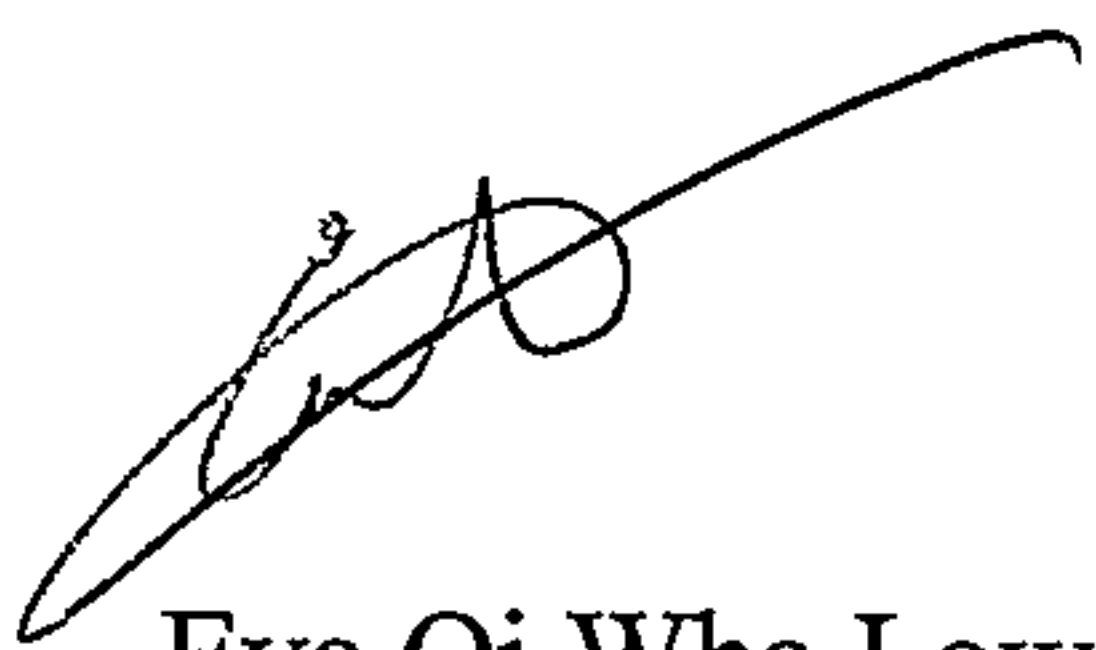
I would like to most of all thank my family for being so patient and encouraging through **all** my years of study.

I would also like to thank the University of Sydney and the Australian Society of Orthodontics Foundation for Research and Education for their generous financial support.

Statement of Originality

This dissertation contains no material which has been accepted for the award of any degree or diploma at any University. To the best of my knowledge and belief, no data presented in this dissertation have been previously published or reported other than the article publications, oral presentations and posters (as reported within) that carry my name as co-author.

The experiments presented in this thesis were performed by myself, except where otherwise acknowledged in the text, in the Department of Orthodontics, Faculty of Dentistry, University of Sydney, Australia.

A handwritten signature in black ink, appearing to be 'Eva Oi Wha Low', written in a cursive style with a long horizontal stroke extending to the right.

Eva Oi Wha Low

August 2003

Publications from this thesis

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Abbreviations

AP-1	activator protein
bp	base pair
cAMP	adenosine 3,5 cyclic phosphate
Cdna	complementary DNA
DCs	dendritic cells
EARR	external apical root resorption
ERK	extracellular signaling-regulated kinase
F-actin	filamentous actin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
H ⁺ ATPase	H ⁺ -adenosine triphosphatase
IL-1	Interleukin-1
IL-1 α R	IL-1 α receptor
INF- γ	Interferon γ
JNK	c-Jun NH2-terminal kinase
MAPK	Mitogen activated protein kinase
MAPKs	mitogen-activated protein kinases
M-CSF	macrophage colony stimulating factor
	MAPK extracellular signaling-regulated kinase (ERK)
MEK	kinase
MMP	matrix metalloproteinases
Mrna	messenger RNA
OCIF	osteoclastogenesis inhibitory factor
ODF	Osteoclast differentiation factor
OPG	osteoprotegerin
OPGL	Osteoprotegerin Ligand
PDL	periodontal ligament
PM	periodontal membrane
PTH	parathyroid hormone
PTHrP	parathyroid hormone-related peptide
RANK	nuclear factor kappa B
RANKL	receptor activator of the NFkB ligand
RGD	Arg-Gly-Asp
SAGE	Serial analysis of gene expression
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
TAK1	TGF- β induced kinase
TGF- β	transforming growth factor β
TNF	tumor necrosis factor
TNFR1	TNF receptor type 1
TNFR2	TNF receptor type 2
TRANCE	Tumor necrosis factor-related activation-induced cytokine

TRAP
UV

tartrate-resistant acid phosphates
ultraviolet

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1. Cellular and molecular control of root resorption

1.1 Introduction

Apical root shortening, a specific type of external resorption, is a complication of orthodontic treatment that is thought to occur, albeit to a small degree, in many patients who undergo comprehensive orthodontic tooth movement. Fortunately it is unusual for this resorption to create a clinical problem. Various biological, mechanical and clinical factors have been considered ¹, however no clear cause for severe root resorption have been found in relation to orthodontic tooth movement.

Root resorption although a common feature of orthodontic tooth movement ^{2, 3}, when the applied force is discontinued or reduced below a certain level the reparative process starts ⁴ and has been recorded as early as during the first week of retention and the healing process increases over time ⁵⁻⁷. Therefore resorption is a condition associated with either a physiologic or pathologic process resulting in a loss of dentin, cementum or bone.

Orthodontic tooth movement is mediated by the coupling of bone resorption on the compressed side of the periodontal ligament (PDL) and bone formation on the stretched side of the PDL ⁸⁻¹⁰.

Differentiation and cellular activities of both osteoblasts and osteoclasts are highly regulated by a wide variety of osteotropic hormones, inflammatory mediators, and growth factors ¹¹.

During bone remodeling, the differentiation, maturation, and function of osteoclasts are regulated by osteoblast-derived factors. One of these, osteoprotegerin (OPG), is a novel secreted member of the tumor necrosis factor (TNF) receptor superfamily that negatively regulates osteoclastogenesis¹²⁻¹⁴. OPG appears to locally and/or systemically inhibit the differentiation of osteoclast precursors into mature osteoclasts, by interrupting the osteoblast –osteoclast precursor interaction^{12, 14, 15}. OPG also inhibits in vitro osteoclastogenesis elicited through distinct signaling pathways stimulated by 1,25(OH)₂D₃, PTH, or IL-11^{12-14, 16}. Another osteoblast-derived factor, the receptor activator of the NFκB ligand (RANKL), has been identified as a member of the membrane-associated TNF ligand family and an important regulatory molecule of osteoclastogenesis^{14, 16-21}. RANKL was found to induce osteoclast differentiation from hemopoietic precursors and stimulate their bone resorptive activity. RANKL is a ligand of OPG and is expressed on the plasma membrane of osteoblasts/stromal cells^{14, 17, 18}. OPG is a soluble decoy receptor for RANKL, and its inhibition of osteoclast differentiation is due to direct binding to a ligand for OPG expressed on osteoblasts/stromal cells^{14, 16, 21-23}. The resorptive activity of osteoclasts induced by soluble RANKL or osteoblasts is completely inhibited by the simultaneous addition of soluble OPG²¹. Therefore, osteoclast differentiation and function are thought to be regulated by the counterbalancing influences of RANKL and OPG.

Investigations into the similarities of controlling mechanisms of cellular activity between dental cells and skeletal tissue cells have recently been actively investigated. Investigations by Kong et al , Rani and MacDougall and Kanzaki et al²⁴⁻²⁷ have shown evidence that dental cells express similar molecules and that these molecules

may have a significant role in the regulation of the balance of dental tissue similar to that of bone.

Physiological root resorption is seen in the process of exfoliation of deciduous teeth and investigations of the expression of cell regulation factors during normal processes of root resorption have also shown the possibility of the importance of such substances such as OPG, and RANKL ²⁸⁻³².

However the role of OPG, RANK and RANKL , have multitudes of roles including regulation of immune, inflammatory responses, lymphocyte regulation, mammary gland formation as well as mammalian evolution of cellular systems. It has been shown to be significant in diseases such as Rheumatoid arthritis, Paget's disease, Osteoporosis, malignant bone diseases (reviewed in Buckley and Fraser 2002, Theil et al. 2002) ^{33, 34}. Interpretation of the important roles of these factors many have systemic and local biological importance relating root resorption, orthodontic tooth movement and bone remodelling.

The review of literature will look at what the current literature presents in terms of

1. Clinical aetiological and associated factors related to root resorption.
2. Cellular processes and physiology in root resorption.
3. What mechanism control and modify root resorption to either enhance or limit root resorption.
4. Cellular processes in bone resorption.
5. Relationship of known and proposed mechanisms that relate the two.

2 Review of literature: Orthodontically induced root resorption

Degrees of root resorption has been classified as

1. Cemental or surface resorption with remodeling. In this process, only the outer cemental layers are resorbed, and they are later fully regenerated or remodeled. This process resembles trabecular bone remodeling.
2. Dentinal resorption with repair (deep resorption). In this process, the cementum and the outer layers of the dentin are resorbed and usually repaired with cementum material. The final shape of the root after this resorption and formation process may or may not be identical to the original form.
3. Circumferential apical root resorption. In this process, full resorption of the hard tissue components of the root apex occurs, and root shortening is evident. Different degrees of apical root shortening are, of course, possible. When the root loses apical material beneath the cementum, no regeneration is possible. External surface repair usually occurs in the cemental layer. Over time, sharp edges may be gradually leveled. Ankylosis is not a common sequel of orthodontically induced inflammatory root resorption.³⁵

2.1 Clinical factors related to external apical root resorption (EARR)

2.1.1 Prevalence

Prevalence of root resorption has varied from 0% ³⁶ to 90% ³⁷. Henry and Weinmann's study in 1951 found 90% of teeth exhibited histological evidence of root resorption in 261 permanent teeth. The number of resorption areas increased with age. 76.8% of resorption occurred in the apical third of the root, with average sizes of resorption areas being 0.73mm in length and 0.1mm in depth. They concluded that teeth undergo normal resorption during its life. Resorption also occurred more on the surfaces facing physiological tooth movement. They also concluded that the size of the resorption cavity was a good indication of the severity while the number of cavities was an indication of the number of incidences that caused the root resorption. In orthodontically treated patients, root resorption has been recorded as high as 93% by Kurol et al. ³⁸. Linge and Linge ³⁹ found up to 17% of treated patients had moderate to severe root resorption which involved greater than 2mm or less than 1/3 of the root length, while up to 5% of treated patients had severe root resorption ⁴⁰. Lupi et al ⁴¹ found increasing incidence of root resorption during orthodontic treatment.

2.1.2 Biological factors

2.1.2.1 Familial considerations

Studies have shown strong familial associations to orthodontically induced root resorption. In 1975, Newman ⁴² suggested family clustering of EARR, although the

pattern of inheritance was not clear. There was no direct evidence for a genetic predisposition until the 1997 report of Harris et al⁴³, who explored the hypothesis of genetic influence on EARR using the sib-pair model; they reported high heritability ($h^2 = 70\%$). Although heritability estimates do not provide information about the number of possible genes contributing to the phenotype, the pivotal report of Harris et al⁴³ indicated that there is probably an important genetic predisposition to EARR. Al-Qawasmi et al^{44, 45} recently reported a genetic marker for those susceptible to EARR. They reported a highly significant evidence of linkage disequilibrium of IL-1B polymorphism with the clinical manifestation of EARR. The analysis indicated that the IL-1B polymorphism accounted for 15% of the total variation of maxillary incisor EARR. Persons homozygous for the IL-1B allele 1 have a 5.6 fold increased risk of EARR greater than 2mm as compared with those who are not homozygous for IL-1B 1 allele.

Racial differences were shown in the study of 868 patients to assess pretreatment factors Sameshima and Sinclair⁴⁶ reported that white and Hispanic patients were more vulnerable than Asian patients to EARR.

2.1.2.2 General health of individuals

Systemic diseases such as hyperparathyroidism, Pagets disease, hypophosphatemia, hypothyroidism and hyperpituitarism have been suggested to be related to root resorption⁴⁷⁻⁵¹. However the publications have been sporadic, Linge and Linge⁵² suggested that hormonal imbalances does not cause but had influences in the process of root resorption. While Goldie and King⁵³ in a study on orthodontically treated hypocalcemic and lactating rats did not support the hypothesis that secondary hyperparathyroidism in response to excessive parathyroid hormone secretion was

responsible for increased root resorption, while Engstrom et al ⁴⁸ found increase in occurrence and severity of root resorption in hypocalcemic rats.

Patients with chronic asthma, both medicated or non-medicated, have shown increased incidence of EARR that is confined to a slight blunting of the maxillary molars ^{54, 55}. They proposed that this finding might result from the close proximity of the roots to the inflamed maxillary sinus and/or the presence of inflammatory mediators in these patients.

Study by Owman-Moll and Kurol ⁵⁶ found that patients with allergies have an increased risk of EARR, although these findings were not statistically significant.

Ong et al ⁵⁷ reported that steroid treated rats displayed significantly less root resorption than controls after orthodontic tooth movement, suggesting that steroid treatment suppressed clastic activity.

2.1.2.3 Gender

There has not been any consensus in the literature in regards to gender susceptibility ^{43, 46, 58}.

In a group of adult (20 years of age) orthodontic patients, Baumrind et al found a greater prevalence of EARR in men than in women. In contrast, Kjar found a greater prevalence of EARR in girls than in boys ^{59, 60}.

2.1.2.4 Dental development

Teeth with open apices have been suggested to be more resistant to apical root resorption ⁶¹. Teeth with incomplete root formation at onset of orthodontic treatment continue to develop roots during treatment, but the roots may not reach expected root length ⁵⁸.

2.1.2.5 Malocclusion

Here again there has been conflicting results from the literature between the relationship of malocclusion and root resorption. VondeAhe did not find a correlation between root resorption and malocclusion classification however others have found that an increase in overjet^{39, 46, 58, 62}, open-bite⁶², and class III malocclusions⁶³ were more susceptible to root resorption. Sameshima and Sinclair however did not find a correlation between overbite and the amount of root resorption⁴⁶.

2.1.2.6 Missing teeth

Levander et al⁶⁴ found a higher risk of apical root resorption during orthodontic treatment in patients with multiple aplasia (with four or more missing teeth), in particular in teeth with abnormal root form and lengthy treatment with elastics and rectangular archwires.

2.1.2.7 Susceptibility of individual teeth due to:

2.1.2.7.1 Position

Brezniak and Wasserstein listed the most frequently affected teeth according to severity ranking the most susceptible to the least as:

1. maxillary lateral incisors
2. maxillary centrals
3. mandibular incisors
4. distal root of mandibular first molars
5. mandibular second premolars
6. maxillary second premolars⁶⁵.

Susceptibility has been associated with the distance moved by the roots during orthodontic treatment and most agree that the maxillary incisor are the teeth most affected by root resorption^{36, 66-69}.

Sameshima and Sinclair found that most root resorption of clinical interest was found in the anterior segment and that the maxillary anterior teeth were more severely affected than the mandibular anterior teeth by a factor of 2, with incisors demonstrating more resorption than canines. The most severely resorbed teeth were maxillary lateral incisors followed by maxillary central incisors, maxillary canines, mandibular canines, mandibular central incisors and mandibular lateral incisors⁴⁶.

2.1.2.7.2 Morphology

Abnormal root shape such as dilacerated, pointed roots have been suggested to be more susceptible to root resorption^{40, 42, 52, 60, 70}. Blunt roots have been shown to be more susceptible to root resorption^{64, 71, 72}. Sameshima and Sinclair⁴⁶ compared the amount of root resorption in normal, blunted, pipette shaped, pointed, dilacerated and incomplete roots after orthodontic treatment and found that dilacerated roots had the most resorption, followed by pipette shaped and pointed roots. However blunted roots showed the least resorption. They suggested in their study that it may be a result of deviant process that caused the abnormal root shape, a genetic component to shape inheritance or disruption of the path of eruption.

Invagination and taurodontism⁶⁰ have been found to be risk factors for EARR on force induction. Normal⁴⁶ as well as small and peg-shaped laterals⁷⁰ have been reported not to be associated with a higher risk of EARR.

In a recent study by Kook et al⁷³ looking at periapical radiographs of 114 patients with 60 peg-shaped and 54 small lateral incisors were measured before and after treatment, they found that there was no significant difference in external root

resorption between peg shaped and normal incisors. However there was a significant difference between small lateral incisor and normal lateral incisors.

2.1.2.7.3 Previous trauma

Traumatized teeth with a history of root resorption have been shown to be more susceptible to further root resorption during orthodontic treatment ^{74, 75}, however those without any signs of resorption did not resorb more than normal teeth ⁷⁶. Linge and Linge ⁵² found that teeth with a history of trauma had a 1.07mm root loss compared with 0.64 mm in untraumatized teeth.

2.1.2.7.4 Endodontic treatment

Some studies suggest that endodontically treated teeth were more resistant to resorption ^{70, 77-79}. While other studies reported a greater incidence of root resorption in endodontically treated teeth ⁸⁰.

2.1.2.7.5 Adjacent Alveolar bone density

Alveolar bone width ^{81, 82} and close proximity of the roots to the cortical plates ^{63, 70} have been studied. Unfortunately, no conclusive results can be drawn from these studies.

It is hypothesized that it is due to the fact that there is a reduced number of marrow spaces in dense alveolar bone and therefore less resorptive cells to remodel bone ⁷⁷.

Goldie and King ⁵³ using calcium deficient rats to decrease bone density, found a reduced amount of root resorption where bone density was decreased.

2.1.2.7.6 Transplantation

Transplanted teeth have been reported to behave similarly to normal teeth. A study by Paulsen and Andreasen ⁸³ showed that transplanted that were rotated orthodontically had some surface resorption and a root shortening on average of 1.2mm.

2.1.3 Mechanical factors

There has been substantial number of studies to try to differentiate types of mechanical influences that would have a greater effect on root shortening during orthodontic tooth movement.

Various aspects have been looked at and these can be divided into:

1. extraction verses non extraction treatment
2. amount of tooth movement
3. type of orthodontic appliance
4. type of orthodontic movement
5. magnitude of orthodontic forces

2.1.3.1 Extraction, non extraction

Sameshima and Sinclair ^{46, 69} found that patients who underwent 4 first premolar extractions had greater resorption than those patients who were treated non-extraction or with upper premolar extractions only. Other extraction patterns of 4 second premolars or mandibular incisor or asymmetric extraction also showed increased resorption. This large sample study contradicted previous studies that showed no difference between patients treated extraction or non extraction ^{66, 84}. Kennedy et al ⁸⁵ found less root resorption in patients treated with serial extractions and without fixed appliances.

2.1.3.2 Amount of tooth movement

Various studies have agreed on the concept that root resorption is directly related to the distance moved by the roots ^{36, 46, 66-69}. Baumrind et al ⁵⁹ in a study analyzing the relationship between upper central incisor displacement measured on lateral

cephalograms and apical root resorption measured on anterior apical films in orthodontically treated adults found that only a relatively small portion of the observed apical resorption could be accounted for by tooth displacement alone, due to the highly variable results seen.

2.1.3.3 Type of Orthodontic appliance

There has been much controversy over the difference in root resorption in association between different types of orthodontic appliances. Standard edgewise techniques have been shown to cause more root resorption than the straight wire technique⁸⁶. No significant difference in the amount of root resorption was found between the SPEED and Edgewise techniques⁸⁷ and between sectional and continuous mechanics⁸⁸. Kinsella found less resorption in the Begg technique however, Almgren found no difference between the two^{76, 89}. Linge and Linge found that use of intermaxillary elastics increased the amount of root resorption, whereas Sameshima and Sinclair found no difference in the amount of resorption with the use of elastics^{46, 52, 69}. The use of Class III elastics for anchorage preparation has been shown to increase resorption of the distal root of the mandibular first molar⁹⁰. The use of rapid maxillary expanders has been shown to cause resorption of maxillary first molars and premolars^{5, 79, 91, 92}, however Sameshima and Sinclair proposed no difference in the amount of root resorption between rapid expansion, slow expansion and no expansion. They also reported no difference between 0.018" slot and 0.022" slot or between stainless steel or titanium archwires.

Removable appliances have been shown to induce less resorption⁵², while there is a suggestion that magnets may stimulate a more physiologic tissue response and decrease the potential for root resorption^{93, 94}.

2.1.3.4 Type of orthodontic tooth movement

Intrusion has been reported as the type of tooth movement that induces the most root shortening^{36, 79, 84, 95-99}. However most other direction of tooth movement has been shown to also cause root resorption^{51, 52, 66, 77, 100-102}.

Reitan and Rygh⁷⁷ and Thilander⁷⁹ suggest there is less root resorption with bodily movement compared with tipping due to less stress distribution along the roots during bodily movement.

There is no agreement whether continuous or intermittent forces produce more or less root resorption, however, it is hypothesized that a pause in treatment with that which occurs during intermittent force application allows the resorbed cementum to heal and therefore is less damaging^{103, 104}.

Maltha and Dijkman¹⁰⁵ compared the amount of root resorption after continuous and discontinuous force application in beagle dogs and showed that discontinuous forces caused less extensive root resorption. Acar et al¹⁰⁶ agreed with this conclusion when 22 human first premolar teeth from 15-23 year old individuals were examined after 9 weeks following the application of a 100gm continuous force and a discontinuous force on each side of the arch. However flaws were inherent in the study, relying on patient compliance to place elastics, while maxillary and mandibular premolars were treated as equivalent.

Owman-Moll et al⁶ examined 32 maxillary first premolars from 11.8-15.8 year olds after the application of a buccally directed continuous and interrupted continuous force of 50g over 4 and 7 weeks and found no difference in the amount or severity of root resorption between the two forces.

2.1.3.5 Magnitude of Orthodontic Forces

Historically, Schwartz considered that forces exceeding the optimal level of 26gm/cm^2 caused periodontal ischemia and therefore may lead to root resorption.

Various animal ^{92, 107, 108} and human studies ^{98, 99, 102, 109} agreed with this concept that increase in force magnitude would increase the amount of root resorption. However conflicting results have been presented by Stenvik and Mjör ⁹⁵ and Owmann- Moll et al ¹¹⁰.

2.1.3.5.1 Animal studies

Dellinger ¹⁰⁷ examined the application of 10g, 50g, 100g and 300g intrusive force to maxillary first premolars of four *Macaca speciosa* monkeys for 60 days and found that root resorption was directly related to force magnitude. The 10g and 50g forces resulted in only slight to moderate root resorption whereas the 100g force gave an increased amount of root resorption and the 300g force resulted in severe resorption. A study applying 50-200g of force in rats reported that light forces produced more rapid tooth movements with less cementum cratering while heavier forces caused slower tooth movement with significant amount of root resorption ¹⁰⁸. Vardimon et al ¹¹¹ found that the predominant factor associated with root resorption was magnitude of force.

2.1.3.5.2 Human studies

Harry and Sims ¹⁰⁹ examined 36 premolar teeth from 11-18 year olds by scanning electron microscopy (SEM) after the application of 50g, 100g, 200g intrusive force over 14, 35, 70 days. The results showed that the distribution of resorbed lacunae was directly related to the amount of stress on the root surface and the rate of lacunae development was more rapid with increasing force. Faltin et al ^{98, 99} applied 50 or 100cN of continuous intrusive force to 12 upper first premolars, the mean age of

subjects were 15.3 years. Transmission electron microscopy showed greater root resorption in those teeth intruded with heavier forces.

Casa et al.¹⁰² applied 300 or 600cN of continuous torque to 28 upper first premolars with a mean age of 13.8years. Teeth were examined at 1, 2, 3, and 4 weeks. Using SEM, it demonstrated that teeth that had been moved with a higher degree of force showed a higher degree of root resorption.

Studies^{95, 96, 110, 112} also show little correlation between force and the degree of root resorption. Owmann-Moll et al,¹¹⁰ examined 32 maxillary premolars from individuals aged 10.1-15.8 years. Force of 50g was applied buccally in 16 teeth and 100g in the other 16. The experimental period was 4 and 7 weeks. The results showed that the severity of root resorption did not differ significantly when the applied force was doubled to 100g and that great individual variation existed regarding both the magnitude of tooth movement and the amount of root resorption. They concluded that individual reactions may have more impact than the increase in force or length of experimental period.

In a subsequent study by the same group, when 50 g force was compared with 200g force applied buccally to first premolars, they found an increase in rate of tooth movement without significant differences in the frequency and severity of root resorption.

2.1.3.6 Treatment duration

Various studies support that the severity of root resorption is directly related to the treatment duration, with longer treatment times creating more resorptive effects,^{1, 2, 38-40, 53, 59, 65, 68, 69, 84, 89, 90, 95, 96, 102, 109, 111, 113-118} a few studies do not support this finding^{36, 39, 66, 101, 119}.

Kvam ¹¹⁶ examined 40 human premolar teeth by SEM from individuals 10-12 years old after application of a 50gm buccally directed force for 5-76 days and found the amount of root resorption increased with time. Reitan ⁹⁶ found similar results in 9-16 year old subjects following application of 25 to 240g of intrusive force. Harry and Sims' ¹⁰⁹ study of 36 human premolar teeth found that severity of root resorption increased with increased treatment time. They concluded that the duration of force was more critical. Kurol ³⁸ showed that the number of resorptions increased with time. Casa et al ¹⁰² used SEM to demonstrate that teeth that had been moved for a longer time period showed a higher degree of root resorption in width and depth.

Multiple radiographical studies also support this theory. Deshields ¹¹⁴ in a radiographic study on root resorption of anterior maxillary teeth in class II division I malocclusions found a statistically significant correlation between treatment time in months and the degree of apical root resorption. Morse ¹¹⁵ found that patients with marked root resorptions had been treated for longer periods. Others also came to similar conclusions ^{84, 117}. Goldin in a radiographic study examining the effect of labial root torque on the maxilla and incisor root apex found that apical root resorption occurred at rate of 12.7% per year. Root resorption was, therefore, greater with longer treatment times. Baumrind et al. ⁵⁹ in a study analyzing the relationship between upper central incisor displacement measured on lateral cephalograms and apical root resorption measured on anterior periapical films in orthodontically treated adults found that treatment time displayed statistically significant association with apical resorption.

However a few studies did not support the concept that increase treatment time would increase root resorption. VondehAhe ⁶⁶ in a radiographic study of orthodontically treated patients and Lilja and Odenrick could not find a correlation between duration

of force application and extent of root resorption. Linge and Linge ¹²⁰ examined radiographically the incident and extent of apical root resorption in maxillary incisors in 719 consecutively treated orthodontic patients. Root lengths were measured in the nearest 0.1mm on standardized intra oral radiographs. They found that treatment time was not closely related to the amount of apical root resorption. Dermaut and DeMunck ³⁶ used periapical X-ray and examined the extent of root resorption after the application of intrusive force on the maxillary central and lateral incisors via a modified Burstone intrusion arch (0.18" Australian wire) engaged in Begg brackets in 20 patients, age ranged from 11- 37 years. Teeth were intruded 20 patients, age ranged from 11-37 average age 15 years for a mean intrusive period of 29 weeks. They found no correlation between the duration of force application and root resorption.

2.1.4 Repair of root resorption

The reparative processes begin when no hyaline tissue is present and / or applied orthodontic force is discontinued or reduced to a certain level ^{77, 96, 121}.

Repair has been recorded 35-70 days after force application ^{95, 109}, within 2 weeks after force cessation ¹²² and during the first week of retention ⁶. The healing process increases with time ^{5-7, 91, 123}. After 5-6 weeks of retention, the repair of root resorption seems to slow down and reach a steady phase ^{6, 92}.

Repair is predominantly characterized by cellular cementum ^{5-7, 92, 119, 123}. Owmann-Moll and Kurol ¹²³ looked at premolars after application of a buccally directed force of 50g which was reactivated weekly for 6 weeks, after which the appliance was passive for 1-7 weeks. They found that most of the reparative cementum was of the cellular type and always covered an initially formed cellular cementum. This was

supported by Vardimon et al in monkeys⁹², by Bosshardt and Schroeder¹²⁴, who suggests that the initial reparative cementum is often acellular, but that continued repair occurs with rapid formation of cellular cementum. Sismanidou and Lindskog¹²² described a healing phase characterized by an initial deposition of acellular cementum within 2 weeks of force cessation, followed by a slower deposition of cellular cementum at a more advanced stages of healing.

Bosshardt and Schroeder¹²⁴ described the attachment of repair cementum to the resorbed roots of permanent teeth. A 1-2µm thick seam of exposed collagen fibrils of residual dentinal matrix lines the resorbed root surfaces. Mononuclear cells repopulate these surfaces and attach to newly produced collagenous matrix fibrils of the exposed dentin matrix. Increasing fibril formation and bundling results in a collagenous fibrous fringe oriented perpendicular to bottom of Howship's lacunae. Following the implantation and establishment of this initial fibrous fringe, a fine granular material indicating the advancing front of mineralization progressively obscures the junctional one between the two matrices. The initially produced matrix is acellular extrinsic fiber cementum, whereas further apposition is with cellular intrinsic fiber cementum.

Different spatial repair patterns have been reported. Animal studies have shown that early repair proceeds from the periphery of the resorption cavity^{92, 125-127}. Brudvik and Rygh^{125, 126} showed in rats that the repair process was characterized by an invasion of fibroblast-like cells from the circumference into active root resorption site. Furthermore, at the same time that repair was occurring in the periphery of the resorption lacunae, active resorption by multi-nucleated odontoclast-like cells took place in the central parts.

Human studies have demonstrated early repair proceeding from the center of the resorption cavity^{5, 123}. Owman-Moll and Kurol¹²³ suggest that the differences

observed in cementum deposition between humans and animals may be due to certain morphological differences between human cementum and the cementum of various animals which is observed in SEM.

Owmann-Moll et al⁶ examined first premolars from individuals aged 11.3-17.3 years, after the application of a buccally directed continuous force of 50g, reactivated weekly over 6 weeks. After which the appliance was passive for 1-8 weeks. The repair patterns were noted as

1. no repair
2. partial repair: part of the surface of the resorption cavity is covered with reparative cementum,
3. functional repair: the total surface of the resorption cavity is covered with reparative cementum without re-establishment of the original root contour
4. anatomic repair: the total surface of the resorption cavity is covered with reparative cementum to such an extent that the original root contour is re-established.

The results of this study showed that partial repair was the most frequent in the first 4 weeks (17-31%) and most commonly seen in cervical third of the root. After 5, 6, 7, and 8 weeks functional repair was common (33-40%). And anatomical repair was seen in the apical third.

3 Cellular processes of root resorption

3.1 Physiology of root resorption

The process of tooth resorption involves and elaborates interaction among inflammatory cells, and hard tissue structures. The process of tooth resorption is believed to be very similar to that of bone ¹²⁸. Injuries to bone, dentine or cementum leads to chemical changes within these tissues. The result is the formation of multinucleated giant cells, also referred as “clast” cells ¹²⁹. The clastic cell is the key cell type responsible for all hard tissue resorptive processes. It is joined by cells such as macrophages and monocytes in resorptive activities. Collectively, these cells orchestrate a complex interplay of molecular biologic events involving cytokines, enzymes and hormones that influences the progression of resorption.

3.2 Monocytes

Monocytes and macrophages, play an important role in bone and tooth resorption ¹³⁰. These cells also play an important role in the development and healing of all wounds ^{131, 132}.

Initially monocytes are recruited to the site of injury or irritation by the release of many pro-inflammatory cytokines. Subsequently, they differentiate into macrophages, whose major role is wound debridement. The migration and recruitment of macrophages into bone are regulated by macrophage chemotactic factors that are derived from bone and tissue breakdown products and are controlled by increased intracellular levels of adenosine 3,5 cyclic phosphate (cAMP) and calcium.

Macrophages or macrophage-like cells are found adjacent to hyalinized tissue in the area of overcompression of the periodontal membrane after the start of experimental tooth movement, and investigators suggest that these cells may play an important role in the removal of hyalinized tissue^{121, 127, 133, 134}. Kurihara¹³⁴ showed that the number of mononuclear cells such as macrophages in the periodontal membrane increases during experimental tooth movement, and that most of them are to be found near the capillaries in the bone-resorption areas. He suggested that these cells might perform the important role of ingesting the necrotic cells or soft tissues. Rygh et al¹³⁵ demonstrated that after the start of experimental tooth movement, macrophages are to be found consistently near blood vessels not only on the pressure side but also on the tension side of the periodontal membrane, and they suggested that macrophages are essential in influencing the remodelling of the membrane either directly or indirectly. Nakamura et al¹³⁶ used antibodies to ED1, ED2, KI-M2R, AND OX6 to identify different subpopulations of macrophages. Their results suggested that after the start of tooth movement OX-6 and ED1 positive cells, which are mostly exudative macrophages, but not ED2 and KI-M2R positive cells which are resident macrophages, may be actively engaged in bone resorption and remodelling of tissues on the pressure side of the periodontal membrane.

3.3 Osteoclasts

Osteoclasts are multinucleated cells formed by the fusion of mononuclear progenitors of the monocyte/macrophage family. They are the principal, if not exclusive, resorptive cell of bone, playing a central role in the formation of the skeleton and regulation of its mass. Bone-forming cells, or osteoblasts have an equally important role in the regulation of bone mass.

3.3.1 Osteoclastogenesis

In vitro maturation of macrophages into osteoclasts requires the presence of marrow stromal cells or their osteoblast progeny ¹³⁷. Only recently has it become clear that accessory cells express the two molecules that are essential and sufficient to promote osteoclastogenesis; macrophage colony stimulating factor (M-CSF) and receptor for activation of nuclear factor kappa B (NF- κ B) (RANK) ligand (RANKL) (also known as OPGL and TRANCE) ¹³⁷.

Although M-CSF is a secreted product, osteoclastogenesis requires contact between osteoclast precursors and stromal cells or osteoblasts ¹³⁷. Thus stromal cells and osteoblasts synthesize a surface-residing molecule, essential for osteoclastogenesis. In 1997, it was found that overexpression or administration of a protein termed osteoprotegerin (OPG), stops osteoclastogenesis in mice. Similarly, animals lacking OPG have accelerated osteoclastogenesis and develop severe osteoporosis ¹². OPG is now known to be a soluble “decoy” receptor that competes with RANK for RANKL ¹⁷. The presence of RANK on osteoclasts and their precursors suggest that osteoclast-differentiating factor, residing on stromal cells, may be RANKL. RANKL is also expressed in abundance by activated T lymphocytes. These cells can directly trigger osteoclastogenesis and are probably pivotal to the such inflammatory diseases such as rheumatoid arthritis ¹³⁸ T-cells are cells that are also influential in inflammatory processes of tooth movement (to be discussed in detail in section 3.11) .

There is an obvious need for a balance between the expression of the stimulator of osteoclastogenesis, RANKL and of the inhibitor, OPG, that dictates the quantity of bone resorbed (Fig. 1).

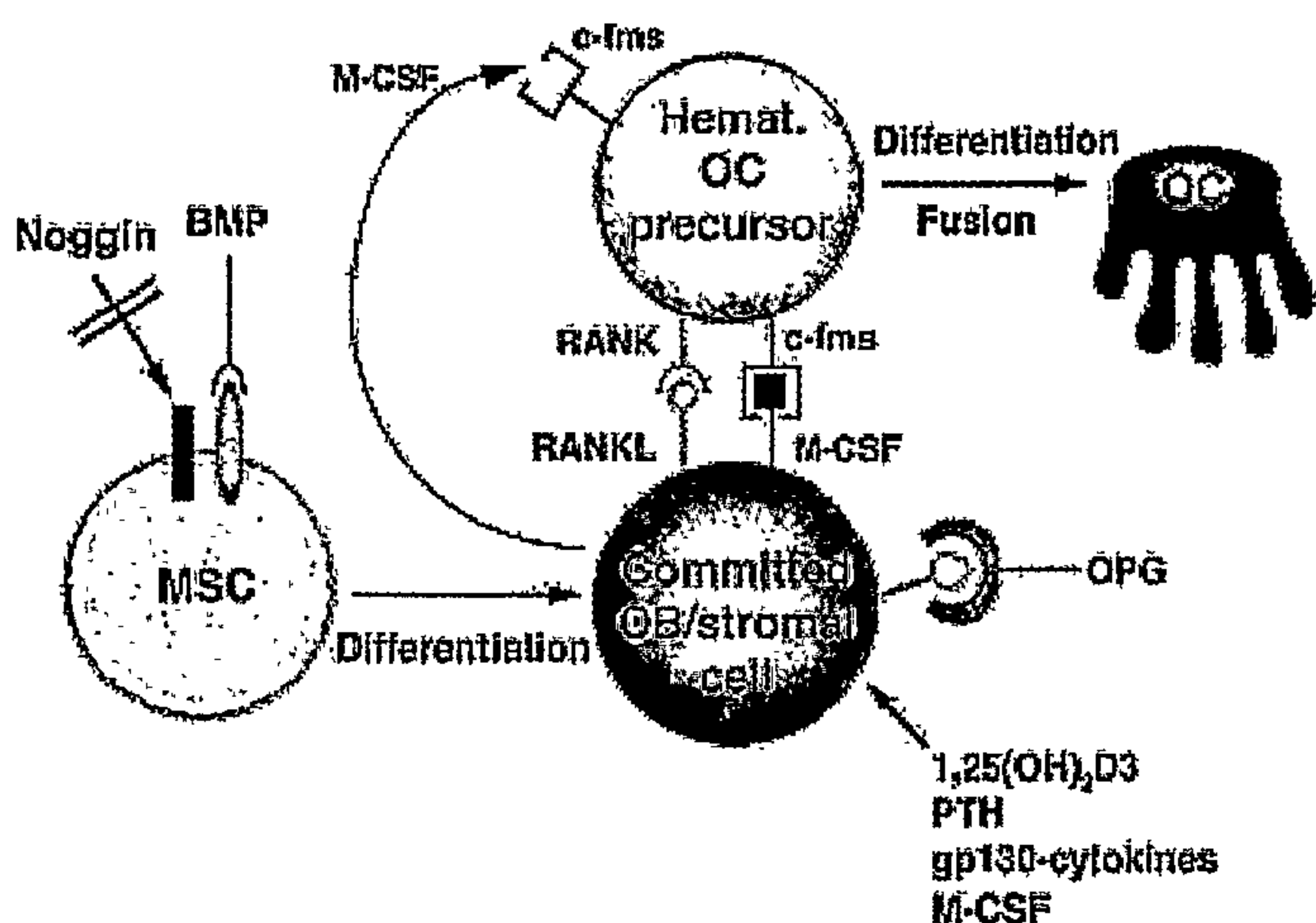


Fig. 1. A model of the cellular interactions for osteoclastogenesis. Mesenchymal stem cells (MSC), the progenitors of stromal cells and osteoblasts, differentiate following exposure to bone morphogenetic protein (BMP). The interaction of BMP with MSCs can be antagonized by proteins like Noggin. Stromal cells and osteoblasts (committed OB/stromal cell) express RANKL on their cell surface. RANKL can be regulated by 1,25(OH)₂D₃ (1,25(OH)₂vitaminD₃), parathyroid hormone (PTH), and cytokines which use gp130 as part of their receptor, like IL-6 and Oncostatin M (gp130 cytokines). RANKL then interacts with its receptor RANK, which is expressed on the surface of osteoclast precursors (Hemat. OC precursor), to induce the terminal differentiation of these cells to mature bony resorbing osteoclasts (OC). Osteoprotegerin (OPG), a decoy receptor, can inhibit the RANKL–RANK interaction by binding RANKL. Soluble M-CSF, secreted by stromal cells or osteoblasts, interacts with its receptor, c-fms to support and enhance osteoclastogenesis. In addition, stromal cells and osteoblasts also express M-CSF on their cell surface; whether this form of M-CSF is active in osteoclast formation remains to be shown. (Horowitz et al ¹³⁹)

The importance of RANKL in osteoclast differentiation emphasizes the central role played by stromal cells. Stromal cells and osteoblasts are the targets of most osteoclastogenic agents that exert their effect by enhancing RANKL expression and thus increasing the quantity of this molecule relative to that of OPG. One such agent is parathyroid hormone (PTH)¹⁴⁰. Disorders of excess PTH are characterized by accelerated osteoclastogenesis, yet osteoclasts lack high-affinity PTH receptors. PTH receptors are known to interact with receptors on osteoblasts and on certain stromal cells that produce the osteoclastogenic factor RANKL. Similarly 1,25-dihydroxyvitamin D₃, the biologically active form of vitamin D₃, is suggested to act

by inducing stromal cell and or osteoblast expression of RANKL ¹⁴¹. RANKL and RANK are members of the tumor necrosis factor (TNF) and TNF receptor superfamilies, respectively. This observation is consistent with clinical data indicating that inflammatory lesions of bone are characterized by abundant osteoclast proliferation. TNF is a potent osteoclastogenic agent and appears to mediate orthopedic implant loosening ¹⁴². The p75 TNF receptor is anti-osteoclastogenesis, while p55 TNF receptor which is bound by bacterial lipopolysaccharide promotes rapid osteoclastogenesis. Therefore the two receptors may reciprocally regulate osteoclastogenesis ¹⁴³. RANKL and p55 TNF receptor activate NF- κ B and c-Jun NH₂-terminal kinase (JNK).

The exact cellular target of TNF and its relationship to RANKL in osteoclastogenesis remains unclear. TNF stimulates osteoblastic cells to express RANKL ¹⁴⁴ and M-CSF ¹⁴⁵, which in turn prompt macrophages to become osteoclasts.

Interleukin-1 (IL-1), like TNF stimulates M-CSF expression by marrow cells, and this effect is inhibited by estrogen ¹⁴⁵.

3.4 RANK RANKL and OPG

3.4.1 Introduction

As alluded to above, the cell-surface member of the tumor necrosis factor (TNF) ligand family, termed receptor activator of nuclear factor κ B ligand (RANKL), or variously termed TNF—related activation-induced cytokine osteoprotegerin ligand/osteoclast differentiation factor (TRANCE, OPGL, ODF) was shown to be central in both osteoclast development and activity ^{17, 146}. Agents that induce bone resorption have been shown to induce the presentation of RANKL on the surface of

osteoblastic cells, which are then able to promote osteoclast formation.^{14, 147} RANKL binds to TNF-receptor superfamily member RANK that is expressed on the surface of osteoclasts and their precursors^{148, 149}. RANK has been shown to be essential for osteoclast formation, and RANK-deficient mice exhibit profound osteopetrosis¹⁵⁰. Likewise, a natural RANKL antagonist, a soluble TNF-receptor family member termed OPG, can inhibit osteoclast formation and bone resorption¹². Overexpression of OPG in mice resulted in an osteopetrotic phenotype, whereas mice in which OPG gene is deleted develop extensive osteoporosis and increased numbers of osteoclasts^{12, 151}. OPG is expressed by a wide variety of cell types including osteoblasts in which its expression is down regulated by many of the same factors that promote bone resorption and RANKL expression¹⁵².

There is now good evidence that the local amount of RANKL relative to OPG is important in osteoclast formation¹⁵³.

3.4.2 Discovery and identification of OPG, RANKL and RANK

In 1997, Simonet et al¹² cloned a new member of the tumour necrosis factor (TNF) receptor family, termed osteoprotegerin (OPG). Interestingly OPG lacked a transmembrane domain and represented a secreted TNF receptor. Tsuda et al 1997¹³ independently isolated a novel protein termed osteoclastogenesis inhibitory factor (OCIF) from conditioned media of human fibroblast culture. The cDNA sequence of OCIF is identical to that of OPG.

OPG is a 401 amino acid protein with four cysteine –rich domains and two death domain homologous regions. The death domain homologous regions share structural features with “death domains” of TNF receptor p55, fas and TRAIL receptors, which mediate apoptotic signals. OPG strongly inhibit osteoclast formation induced by

either $1\alpha,25(\text{OH})_2\text{D}_3$, PTH, prostaglandin E2 or IL-11 in mouse co-culture¹²⁻¹⁴. Analyses of transgenic mice expression of *Opg* and animals injected with OPG have demonstrated that this factor suppresses osteoclastic bone resorption, resulting in increased bone mass^{12, 14}. In contrast *Opg* knockout mice exhibited severe osteoporosis due to enhanced osteoclastogenesis¹⁵¹.

RANKL which binds OPG and regulates osteoclastogenesis was identified shortly after OPG was discovered^{14, 17}. RANKL is a type II transmembrane protein which had been previously shown to be expressed on the cell surface of activated T cells and called TNF-related activation-induced cytokine (TRANCE)¹⁵⁴. RANKL is a 317 amino acid polypeptide that has significant homology with other TNF ligand family members including TRAIL (20–35% homology), CD40 ligand (28% homology), and FAS ligand (19% homology)^{14, 17, 146}. Human RANKL has no signal peptide, has an N terminal cytoplasmic domain (residues 1–48), a transmembrane domain (residues 49–69, and an extracellular domain (residues 70–317) that contains a ligand binding site (residues 158–317)^{14, 17, 146}. RANKL exists functionally as both a membrane-bound protein (40–45 kDa) and as a soluble protein (31 kDa)¹⁷. The soluble form is cleaved from the cell surface specifically by metalloprotease-disintegrin TNF- α convertase (TACE). The existence of a biologically active soluble form of RANKL could explain previous data showing that conditioned medium from cytokine activated stromal cells or osteoblasts could induce osteoclast formation in the absence of direct cell-to-cell contact. The human RANKL gene has been mapped to chromosome 13q14^{146, 154}.

As expected, mRNA for RANKL is expressed at high levels in bone, bone marrow, and in lymphoid tissue including fetal liver, lymph nodes, spleen, and thymus. Lower

levels can also be detected in heart, lung, thyroid, and placenta ^{17, 22, 146, 154}. In developing bone, RANKL is expressed in early mesenchymal cells which are in close proximity to cartilaginous anlagen and in hypertrophic chondrocytes ¹⁷. In adult bone, RANKL mRNA is expressed in high levels in the growth plate and periosteum ¹⁷. Steady state levels of RANKL mRNA are increased by treating cells with parathyroid hormone,

$1\alpha 25-(OH)_2$ vitamin D₃, dexamethasone, IL-1, IL-11, Oncostatin M, and PGE₂ ^{14, 140, 144, 147, 152}. In contrast, TGF β suppresses expression ¹⁵⁵.

The research group who cloned RANKL cloned a new member of the TNF receptor family termed RANK from a cDNA library of human dendritic cells (Andersen 1997) ¹⁴⁶. The mouse RANK cDNA encoded a type 1 transmembrane protein of 625 amino acids residues (Fig.2). A genetically engineered soluble form of RANK, and extracellular domain of RANK, inhibited ODF-mediated osteoclast formation ¹⁵⁶. Polyclonal antibodies against the extracellular domain of RANK induced osteoclast formation in pure spleen cell culture in the presence of M-CSF ¹⁵⁶. In contrast the Fab fragment of the antibody blocked the binding of ODF to RANK, causing the inhibition of the ODF-mediated osteoclastogenesis ¹⁵⁶. These results suggest that RANK is the sole signally receptor for ODF in inducing osteoclast differentiation ¹⁵⁷.

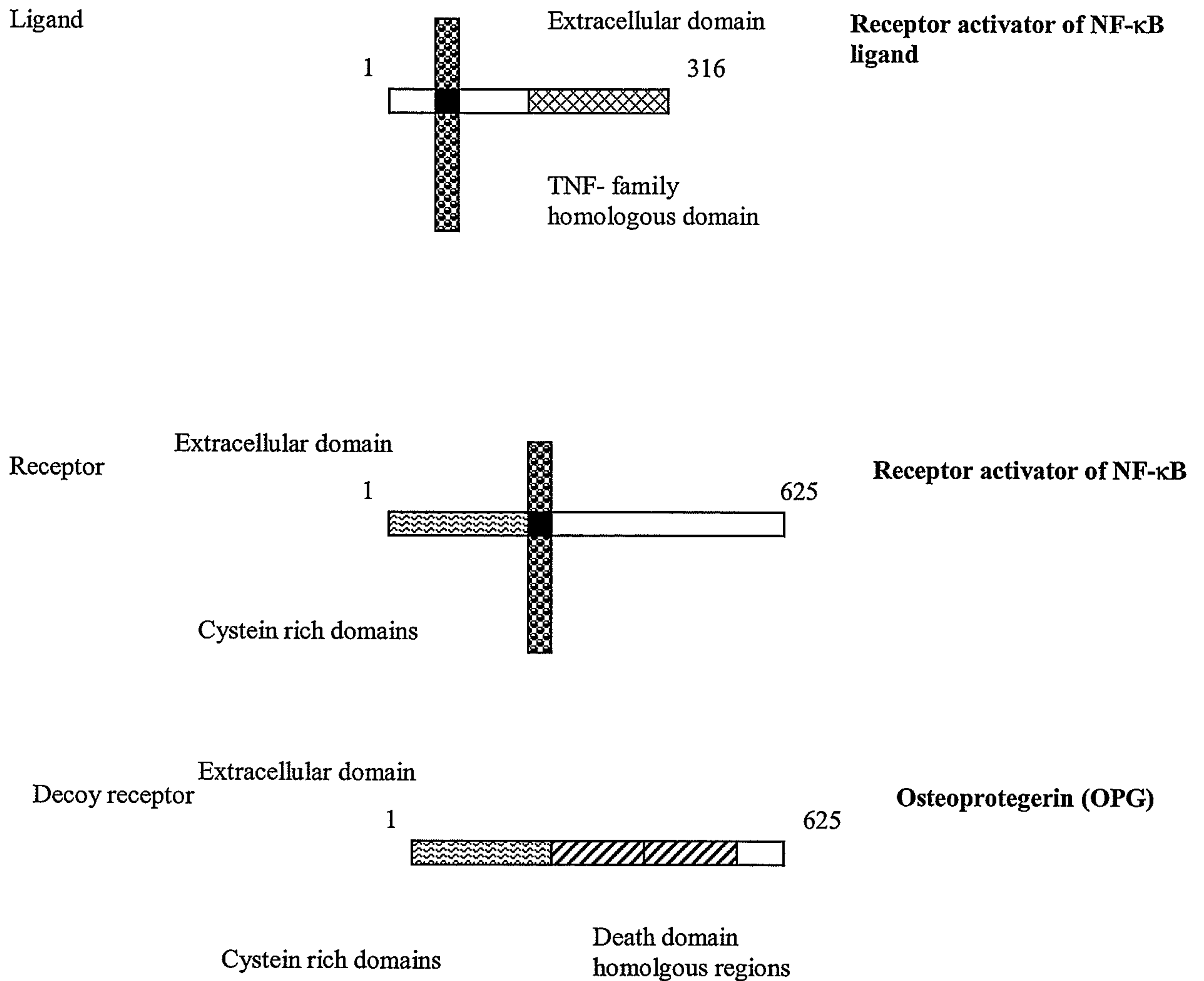


Figure 2. A schematic representation of the TNF ligand, receptor and decoy receptor

(From Suda et al ¹⁵⁸)

3.4.3 Signal transduction of TNF family members

TNFα induces its biological response through two cell surface receptors termed TNF receptor type 1 (TNFR1, p55) and TNF receptor type 2(TNFR2, p75). TNF-receptor

associate factor 2 (TRAF2) has been shown to be an adaptor protein for mediating the signals induced by TNFR1 and TNFR2^{159, 160}. In addition the binding of TNF α to TNFR1 triggers apoptosis in many cell types.

ODF induces its signals through RANK. Recent studies have demonstrated that the cytoplasmic tail of RANK interacts with TRAFs 1,2,3,5, and 6 (Fig. 3)¹⁵⁴.

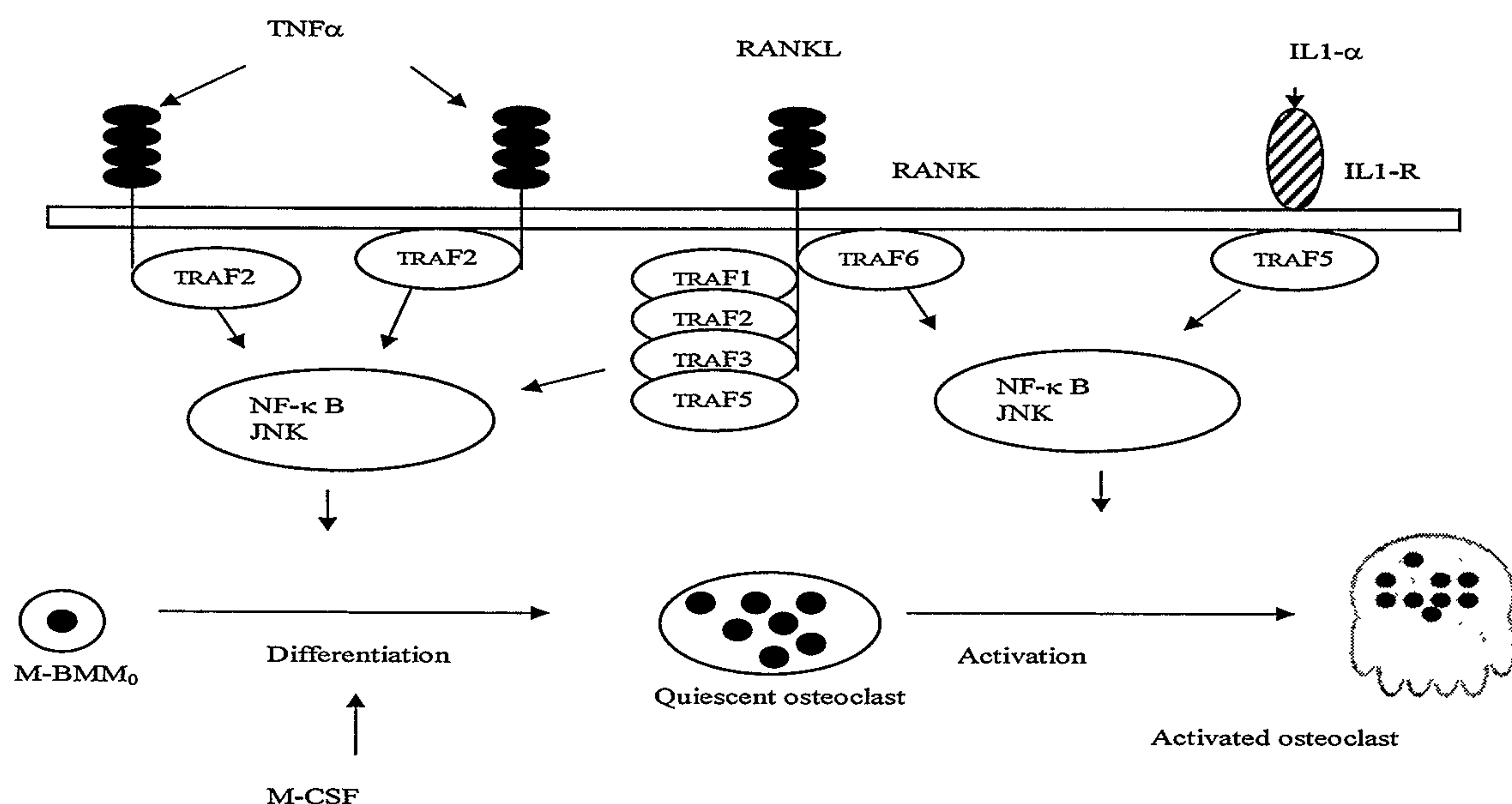


Figure 3. Schematic representation of ligand receptor systems in osteoclast differentiation and function regulated by TNF α , ODF and IL-1 α .

TNF α and ODF independently stimulate osteoclast differentiation. Osteoclast differentiation induced by TNF α occurs via TNFR1 and TNFR2 expressed by osteoclast precursors. ODF induces osteoclast differentiation through RANK-mediated signals. M-CSF is common factor required for both TNF α - AND ODF-induced osteoclast differentiation. Activation of osteoclasts is induced by ODF and IL-1 α through RANK and type 1 IL-1 α receptor (IL-R) respectively. TRAF6 appears to be involved commonly in osteoclast activation induced by ODF and IL1 α .

Common signalling cascades such as NF- κ B and JNK activation may be involved in the differentiation and activation of osteoclasts induced by TNF α ., ODF/ RANKL and IL-1 α . (Suda et al ¹⁵⁸)

The interaction of these TRAFs with RANK appears to be involved in osteoclast differentiation and activation. IL-1R is shown to use TRAF6 as a signal transducing molecule ¹⁶¹. Lomaga et al and Naito et al ^{162, 163} independently reported that *Traf6* knockout mice developed severe osteopetrosis. It was shown that in spite of the presence of similar numbers of osteoclasts between TRAF6 knockout mice and wild-type mice, osteoclasts in *Traf6* knockout mice failed to form ruffled borders ¹⁶².

Mitogen activated protein kinase (MAPK) related TGF- β induced kinase (TAK1) along with the TRAF-binding adapter protein TAB2 have been detected in activated receptor complexes ^{164, 165} dominant interfering mutant forms of TAK1 inhibit RANKL- mediated activation of both IKK1/2 and JNK1 suggesting that TAK1 is important in activation of NF- κ B and AP-1 ¹⁶⁶.

Stress-activated protein kinase p38 is also involved in mediating key signals induced by RANK and is apparently activated via phosphorylation by MKK6 ^{164, 165}.

3.4.4 Regulation of RANK induced osteoclastogenesis

The RANK signalling pathway is negatively controlled by OPG *in vitro* and *in vivo*. ^{12, 14} In addition to this, chemical inhibitors of MEK1 lead to increased osteoclastogenesis *in vitro*, suggesting that activation of ERK and Src pathways can also negatively regulated osteoclastogenesis ¹⁶⁷. There is also evidence for feedback mechanisms that switch off the RANK signalling pathway once it is activated. Induction of osteoclastogenesis by RANKL leads to the induction of interferon - β

which is secreted and acts in an autocrine fashion to down regulate the expression of c-Fos, a critical factor involved in osteoclast development¹⁶⁸. Interferon γ (INF- γ) also has a negative effect on this pathway. Binding of INF- γ to its receptor leads to degradation of TRAF6, and can have an inhibitory effect on osteoclastogenesis in vitro¹⁶⁹. The cytokine IL-4 has also recently been shown to negatively regulate osteoclastogenesis and this process is controlled downstream by signal transducer and activator of transcription signalling within the osteoclast¹⁷⁰. The binding of calcitonin to its receptor has been known to dampen osteoclast activation and is the basis for using it as a therapeutic although its molecular mechanism of action is unclear.

3.4.5 Hormonal regulation of bone resorption

Certain hormones, cytokines and humoral factors produced in distant organs can also influence bone density and calcium homeostasis locally by inducing RANKL expression within the bone cells. Calciotropic hormones and pre-resorptive cytokines have been shown to up regulate messenger RNA expression of RANKL in osteoblast cell lines and primary cell cultures^{23, 170, 171}. OPG, which blocks osteoclastogenesis induced by RANKL, can inhibit osteoclast formation and bone resorption induced by treatment with calciotropic factors, indicating that the RANK signalling pathway in osteoclasts integrates diverse humoral signals that regulate bone resorption and calcium homeostasis. Consistent with this hypothesis, RANK- deficient mice are resistant to bone resorption induced by TNF- α , IL-1 β , 1,25(OH)₂, vitamin D and parathyroid hormone (PTH)-related peptide (PTHrP), which excluding PTH are major calciotropic factors that are known to induce increases in bone resorption and serum hypercalcemia¹⁷². The T cell is also an important source of RANKL in the bone¹³⁸. Activation of T cells in vitro and in vivo leads to increased osteoclastogenesis and

bone resorption, suggesting that acute and chronic inflammatory states and certain leukaemias contribute to pathologic bone loss ²⁴.

Humoral factors that decrease bone resorption and increase density, such as oestrogens have a converse effect on the coupling between the osteoblast and osteoclast. OPG expression is increased and/ or RANKL expression is decreased, leading to decreased activation of RANK and subsequently the numbers of activated osteoclasts in the bone. Osteoblast and marrow stromal cells, as well as T-cells, can act as a key local responding unit within the one that interpret physiological cues that impact bone remodelling rates , calcium release and bone density.

3.4.6 OPG expression and regulation

Compared with that of RANKL, steady state levels of OPG mRNA are higher and have a wider tissue distribution that is not restricted to bone or immune tissues ^{12, 14, 173}. High levels of OPG mRNA have been detected in lung, heart, kidney, liver, stomach, intestine, skin, brain and spinal cord, thyroid gland and bone. ^{12, 14, 173} Levels of OPG mRNA and protein in osteoblastic lineage cells are increased by the cytokines IL-1 α , IL-1 β ¹⁷⁴ TNF- α and TNF- β ^{174, 175} by the osteoblast differentiating factors 1 α , 25-(OH)2D3 and bone morphogenetic protein 2 ¹⁷⁴ and by the antiresorptive agents estrogen ¹⁷⁴ and TGF- β ^{155, 176}. OPG mRNA and protein levels are decreased by glucocorticoids ¹⁴⁴, PGE2 ¹⁷⁵ and by the pure estrogen receptor antagonist ICI 182, 780 ¹⁷⁴.

3.4.6.1 Biological effects

OPG acts as a soluble secreted receptor for RANKL that prevents it from binding to and activating RANK on the osteoclast surface. The biological effects of OPG on

bone cells are opposite to that of RANK, including inhibition of the terminal stages of osteoclast differentiation,¹²⁻¹⁴ suppression of the activation of mature osteoclasts,^{17, 177, 178}, and the induction of apoptosis¹⁵.

By using a spleen cell/bone marrow stromal cell coculture system, Simonet et al¹² have shown that the human recombinant OPG dimer inhibited osteoclast differentiation. Tsuda et al¹³ reported that osteoclast differentiation was inhibited at concentrations from 1-40ng/ml. Treatment with OPG also inhibited the osteoclastogenesis induced by treatment with 1α , $25(\text{OH})_2\text{D}_3$, PGE2, PTH, IL-1, IL-11^{13, 177}.

In addition to blocking osteoclast formation OPG also inhibits osteoclastic pit formation of mature osteoclasts. Inhibition of osteoclast activation as opposed to differentiation requires higher concentrations of OPG^{18, 177}. Recently OPG has been implicated to have effects on fibroblast proliferation to act as a soluble receptor for TRAIL to inhibit TRAIL-induced apoptosis¹⁷⁹.

Transgenic mice overexpressing OPG have severe osteopetrosis with narrowing of the bone marrow cavities, resulting in splenomegaly due to compensatory extramedullary hematopoiesis. In contrast to OPG knockout mice¹³⁸ transgenic mice overexpressing OPG lacked defects of tooth eruption, lymphocyte development or lymph node organogenesis¹². A possible explanation for the differences in these phenotypes may be that OPG gene was completely inactivated during embryogenesis whereas the OPG trans gene was activated only after birth, thus permitting RANKL effects before birth. Targeted ablation of OPG resulted in mice with severe osteoporosis¹⁵¹. Bone density of OPG deficient mice was also significantly less¹⁵¹. Interestingly OPG deficient mice also had profound calcification of the large arteries by 2 months of age that progressed to marked medial calcification of the aorta and renal arteries, profound

intimal and medial proliferation and partial aortic dissection by 4 months. Because OPG is expressed in the media of large arteries in the wild type mice, it may play a protective role in vascular biology¹⁵¹.

3.5 Mechanism of bone resorption

Bone resorption is a multistep process initiated by the proliferation of immature osteoclast precursors, the commitment of these cells to osteoclast phenotype and finally degradation of the organic and inorganic phases of bone by the resorptive cells. At first approximation, the osteoclast resembles the foreign body giant cell, which is a macrophage polykaryon formed in response to exogenous particulate matter. The osteoclast is able to polarize on bone and then forms a ruffled membrane. This complex infolding of the plasma membrane juxtaposed to the matrix is the osteoclast's resorptive organelle and appears only when the cell is attached to bone. The ruffled membrane probably represents the transport of acidifying vesicles along microtubules and their polarized insertion into the plasma membrane, an event similar to exocytosis^{104, 180}.

The initial event in bone degradation is the attachment of osteoclasts to target matrix. Once attached to bone, the cell generates an isolated extracellular microenvironment between itself and the bone surface¹⁸¹. The intimacy between osteoclasts and bone, required for resorption is reflected by the matrix attachment or sealing zone. This structure, is a rich filamentous actin (F-actin) and largely devoid of organelles, is organized as a ring surrounding the ruffled membrane¹⁸². The F-actin in the sealing zone localizes in punctate plasma membrane protrusions known as podosomes¹³⁷. In addition to F-actin, these structures contain proteins such as vinculin, talin, and α -

actinin, which link matrix-recognizing integrins to the cytoskeleton ¹⁸³. The appearance of the podosomal ring, like the ruffled membrane, parallels bone resorption. The ring appears when the osteoclast is immobilized on and engaged in degrading bone and disappears after osteoclast detachment and motility ¹⁸².

Bone consists largely of type 1 collagen and of non collagenous proteins containing a mineral phase of substituted hydroxylapatite. Dissolution of the inorganic phase of bone precedes matrix degradation ¹⁸⁴. Bone mineralization involves acidification of the isolated extracellular microenvironment, a process mediated by a vacuolar H⁺-adenosine triphosphatase (H⁺-ATPase) in the cell's ruffled membrane, which is similar to the proton pump in the intercalated cells of the renal tubule ^{184, 185}. The intra-osteoclastic pH is maintained, in the face of abundant proton transport, by an energy-independent Cl⁻/HCO₃⁻ exchanger on the cell's antiresorptive surface ¹⁸⁶. Finally electroneutrality is preserved by a ruffled membrane Cl⁻ channel, charge-coupled to the H⁺-ATPase ¹⁸⁷. The result of these ion transporting events is secretion of HCl into the resorptive microenvironment, prompting a pH of approximately 4.5 ¹⁸¹. This acidic milieu first mobilizes bone mineral; subsequently, the demineralized organic component of bone is degraded by a lysosomal protease, cathepsin K ¹⁸⁸. The products of bone degradation are endocytosed by the osteoclast and transported to and release at the cell's antiresorptive surface ¹⁸⁹.

The degradation of the organic matrix is accomplished by three groups of proteinase enzymes: collagenases and matrix metalloproteinases (MMP) which act at neutral or just below neutral pH (7.4) ¹⁹⁰, and cysteine proteinase family which act at an acidic pH ¹⁹¹. The cysteine proteinases appear to work closer to the ruffled border where the pH is more acidic, while the collagenases appear to be active at the resorbing bone

surface, where the pH is closer to neutral because of the buffering capacity of the dissolving bone salts. Cysteine proteinases are secreted directly by the osteoclasts into the clear zone via the ruffled border. While the source of collagenases remain in question.

Metalloproteinases may also play a role in osteoclast function. Mice carrying a mutation in the site in type 1 collagen that is targeted by the neutral collagenase has attenuation in resorption ¹⁹².

The functional cycle of the osteoclast consists of episodes of matrix adherence followed by detachment and movement to a new site of bone degradation ¹⁸². The signals that arrest the process of bone resorption is not well known. A proposed theory is that the plasma membrane receptor sensing of high calcium levels within the resorptive space prompts the withdrawal of the osteoclasts from the bone surface and the termination of resorption at that site. More evidence though is suggesting that the bone sparing effects of antiosteoporosis agents such as estrogen ^{193, 194} and bisphosphonates ¹⁹⁵ causes a stimulation of osteoclast apoptosis. In the case of oestrogen, osteoclast death is mediated by transforming growth factor β (TGF- β) ¹⁹³, whereas bisphosphonates promote apoptosis by inhibiting the melavonate pathway ¹⁹⁵.

3.6 Genetic regulation of osteoclast

Information on the genetic regulation of osteoclasts has been mainly derived from the study of bone diseases. Osteopetrosis is a family of diseases characterized by a marked enhancement of bone mass that is caused by osteoclast dysfunction. The osteoclast is derived from the monocyte/macrophage lineage ¹³⁷, and it therefore follows that genes regulating the maturation of these mononuclear cells would also

modulate the osteoclast. One example is the *PU.1* gene, which encodes a transcription factor essential for macrophage differentiation ¹⁹⁶. Mice deficient in PU.1 lack macrophages as well as osteoclasts and therefore develop osteopetrosis. The transcription factor c-Fos is also required for osteoclast differentiation. The deletion of C-Fos stops the expression of Fra-1, which like c-Fos is a member of the AP-1 (activator protein) transcription factor family. Inactivation of c-Fos in mice results in arrested osteoclastogenesis and osteopetrosis that can be rescued by marrow transplantation ¹⁹⁷ and by transgene expression Fra-1 ¹⁹⁸. C-Fos deficient mice have elevated numbers of marrow macrophages ¹⁹⁷. Therefore c-Fos serves to commit hematopoietic precursors to become osteoclasts instead of mature macrophages and it exerts its effect distal to PU.1.

AP-1 transcription factor in osteoclast differentiation is critical in that overexpression in macrophages of another member of the family, c-Jun, mutated to render it nonactivatable by JNK, prevents their differentiation into osteoclasts.

The transcription complex NF- κ B is also essential for osteoclastogenesis, because mice lacking its p50 and 52 subunits develop osteopetrosis caused by the arrested generation of osteoclasts from macrophages ¹⁹⁹.

RANKL and M-CSF ^{14, 24} are essential for osteoclastogenesis, and mice lacking either protein fail to generate osteoclasts and hence develop osteopetrosis. The cellular defect in these mutants, lies not in the osteoclast precursor but in the stromal microenvironment necessary for osteoclastogenesis.

Now at least 24 genes or loci (Figure 4) have been shown to positively and negatively regulate osteoclastogenesis and osteoclast activation, based on naturally occurring mutations or targeted knockout mutations in rodents and humans ^{200, 201}. Disruption of these genes blocks the development and or function of the mature osteoclast.

Some genes act during the formation and or survival of the osteoclast precursor cell (*PU.1*, and *op/CSF-1*) whereas other genes mediate either the ability of the precursor cell to undergo differentiation (*RANK*, *p50/p52*, *rel* and *fos*) or the adherence and lytic function of the mature osteoclast (*src*, *oc/Tcirg* and *CATK*)²⁰².

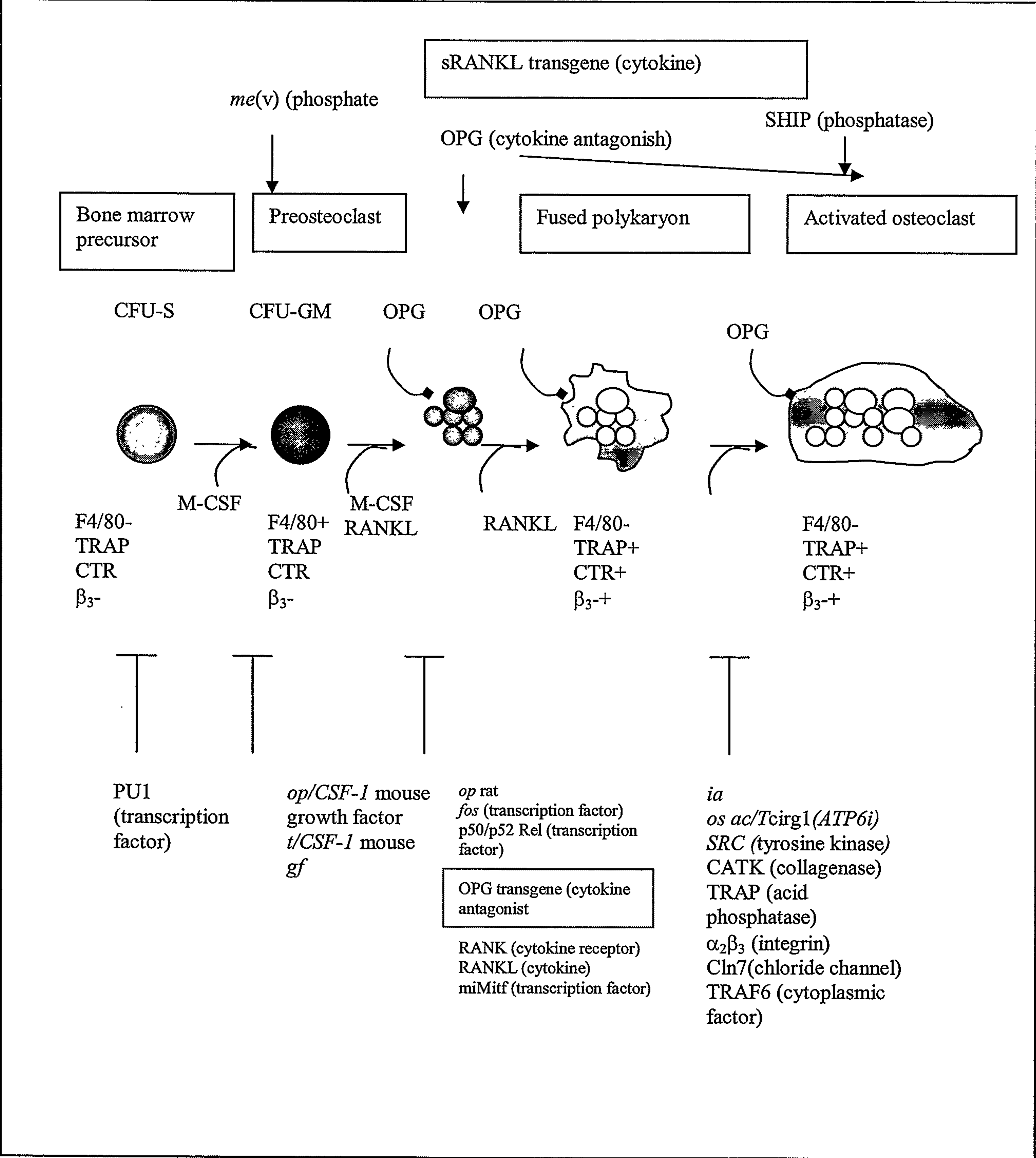


Figure 4 Osteoclastogenesis.

Development schema of haematopoietic precursor cell differentiation into mature osteoclasts, which are fused polykaryons arising from multiple (10–20) individual cells. Maturation occurs on bone from peripheral bloodborne mononuclear cells with traits of the macrophage lineage. M-CSF (CSF-1) and RANKL are essential for osteoclastogenesis, and their action during lineage allocation and maturation is shown. OPG can bind and neutralize RANKL, and can negatively regulate both osteoclastogenesis and activation of mature osteoclasts. Shown below are the single-gene mutations that block osteoclastogenesis and activation. Those indicated in *italic* font are naturally occurring mutations in rodents and humans, whereas the others are the result of targeted mutagenesis to generate null alleles. Shown above are the single-gene mutant alleles that increase osteoclastogenesis and/or activation and survival and result in osteoporosis. Note that all of these mutants represent null mutations with the exception of the OPG and sRANKL transgenic mouse overexpression models (in blue-outlined boxes).²⁰²

3.7 Other significant biological roles of OPG, RANKL and RANK

Prior to its discovery as a major effector in bone RANKL had been found to be expressed by activated T lymphocytes and was therefore named TNF-related activation induced cytokine (TRANCE)¹⁴⁶.

Members of the tumor necrosis factor superfamilies of ligands and cell-surface receptors regulate immune function, and most TNF/TNFR superfamily proteins, such as FASL/FAS, CD40L/CD40, TNF/TNFR, or LTBeta/LTBetaR, to name a few, are expressed in the immune system, where they coordinate immune cell homeostasis, activation-induced cell death, T cell priming, functions, and survival of dendritic cells, or the formation of germinal centers and lymphoid organs such as Peyer's patches and lymph nodes^{203, 204}. Recently, novel members of this large family have been identified that have critical functions in immunity and couple lymphoid cells with other organ systems such as bone morphogenesis and mammary gland formation

in pregnancy. RANKL, RANK and OPG proteins have become principal targets in development of new therapies for osteoporosis, arthritis, or bone metastases in cancer. RANK-L is also responsible for regulating T cell/dendritic cell communications, dendritic cell survival, and lymph node organogenesis. Production of RANK-L by activated T cells can directly regulate osteoclastogenesis and bone remodeling, and it explains why autoimmune diseases, cancers, leukemias, asthma, chronic viral infections, and periodontal disease result in systemic and local bone loss. In particular, RANK-L appears to be the pathogenetic principle that causes bone and cartilage destruction in arthritis²⁰².

Inhibition of RANK-L function via the natural decoy receptor osteoprotegerin prevents bone loss in postmenopausal osteoporosis and cancer metastases and completely blocks crippling in a rat model of arthritis.

3.7.1 The Role of RANK-L and RANK in the Immune System

At around the same time we had the first evidence that RANK-L might play a role in osteoclast development, RANK-L (TRANCE) was independently cloned by two other groups as a molecule expressed on the surface of activated T cells. Both soluble and membrane-bound RANK-L is produced by activated CD4 and CD8 T cells^{24, 154}. RANK-L is also expressed in lymph nodes, spleen, thymus and intestinal lymphoid patches¹⁷, and immature CD4 and CD8 thymocytes¹⁴⁶. RANK-L expression in T cells is induced by antigen receptor engagement and is regulated by calcineurin, ERK1/ERK-2, and PKC-regulated signaling pathways^{24, 154}. RANK is expressed on the surface of dendritic cells (DCs), mature T cells, and hematopoietic precursors^{146, 154, 205}. In addition, OPG was found in a screen to identify novel genes expressed in follicular dendritic cells. OPG can be found on the cell surface of DCs probably by

capturing of soluble OPG the cell membrane via binding of a hyalurinic acid binding region present in OPG ²⁰⁶. Thus, like the interactions between CD40-L and CD40, or CD28 and CD80/CD86, the binding of RANK-L to RANK can regulate DC functions, T cell activation, and T cell-DC communication in vitro ¹⁴⁶. Moreover, OPG may modulate this interaction.

Lymph node organogenesis during the initial analyses of rank -/- and later of rank mutant mice, a completely unexpected phenotype became evident: rankl-/- and rank -/- mice displayed complete absence of all lymph nodes ^{138, 150}.

3.7.2 T cells and Bone turnover

It has been postulated that T cells modulate bone turnover via RANKL²⁴. But since mice that lack T cells still have normal bone cavities and tooth eruption, T cells are probably not required for normal bone homeostasis. However chronic systemic T cell activation such as in autoimmune diseases, viral infections or local inflammation within the bone due to metastasis, infections and fractures, or joint inflammation in arthritis all probably attract T cells that then actively participate in bone remodeling via production of RANKL.

3.7.3 Bone Loss, Mammary Gland formation and mammalian evolution

RANKL and OPG as mentioned are influenced by multiple hormones. Intriguingly, expression of RANKL and OPG are also strongly influenced by the female sex hormones progesterone and estrogen and by hormones involved in reproduction and lactation such as prolactin and parathyroid hormone-related peptide ²⁰⁷. Interestingly mice lacking RANKL or its receptor RANK fail to form lobuloalveolar mammary

gland structures during pregnancy and show a complete block in formation of a lactating mammary gland leading to death of new born pups ²⁰⁸. It is hypothesized that RANKL and RANK regulate skeletal calcium, and are essential for the formation of the lactating mammary gland, the organ required for transmission of maternal calcium to neonates in mammalian species. The data provided a novel function of TNF and TNFR family proteins and a new paradigm in the formation of lactating mammary gland.

3.8 Resorption of bone during tooth movement

3.8.1 Odontoclasts

It is unknown precisely whether osteoclasts and tooth resorbing (dentinoclasts, odontoclasts and cementoclasts) are the same cell, but a number of similarities do exist. Odontoclasts are smaller, have a ruffled border, also contain a microfilamentous cytoskeleton, contain fewer nuclei than osteoclasts, and have smaller or no clear zone ¹²⁸. Both cells have similar enzymatic properties and intense tartrate-resistant acid phosphatase activity.

Cells identified as resorptive, lodged *in vivo* in Howship's lacunae in enamel, dentine or cementum, have the same cytological features characteristic of osteoclasts ^{209, 210}.

They are polarized with respect to the dental tissue, have a ruffled border within an annular clear zone that is closely adherent to the hard tissue matrix, are usually multinucleate and contain abundant mitochondria and lysosomes. Proof that the same cell type resorbs all three dental tissues *in vivo* is obtained from those odontoclasts

whose resorptive apparatus spans the junction between cementum and dentine, dentine and enamel or even all three tissues. An odontoclast may also have separate ruffled borders active on different tissues ²¹¹.

Mononuclear odontoclasts participate in tooth resorption. However the majority of odontoclasts (94%) that form lacunae on the dentin are multinucleated, having 10 or fewer nuclei. Oligonuclear odontoclasts (cells with fewer than five nuclei) resorb more dentin per nucleus than do cells with a higher number of nuclei ²¹². Both osteoclasts and tooth resorbing cells have receptors for vitamin D, as local injection of 1,25-(OH)₂ D₃ results in both an increase in osteoclast number and root resorption ²¹³. Despite similarities, there is evidence to show that odontoclasts and osteoclasts are distinct populations of cells. Odontoclasts can differentiate and resorb under prostanoid inhibition while osteoclastic resorption is inhibited ²¹⁴.

Little information is available on odontoclast precursors or their differentiation into functional odontoclasts ²¹⁵. Funenzalika et al 1999²¹⁶ looked at the cytodifferentiation of odontoclasts using tartrate-resistant acid phosphates (TRAP) activity as a histochemical marker, they identified mononuclear precursor cells and could observe morphological and functional differentiation into mature multinucleate odontoclasts.

They observed four stages:

1. preresorption stage
2. early resorption stage
3. later resorption stage and
4. final resorption stage

Sahara et al in 1992 and 1996 ^{215, 217} suggested that cells appearing in the dental pulp of human deciduous teeth prior to shedding might be precursor cells of odontoclasts.

Work by Fuenzalida et al ²¹⁶ also showed TRAP positive mononuclear cells in the dental pulp were precursor cells of the odontoclasts.

Fuenzalida et al ²¹⁶ observed that the membrane specialization of odontoclasts, that is the development of a clear zone and ruffled border is induced following their contact with resorption surface and that the multinucleation of odontoclasts takes place only after their attachment to the resorption surface. Sahara et al reported a similar pattern of ruffled borders in odontoclasts of human deciduous teeth and suggested that membrane specialization and multinucleation of odontoclasts would take place rapidly or simultaneously after the attachment to the predentine surface, since the initial ruffled border was usually found in odontoclasts that had two or three nuclei. However, mononuclear odontoclasts with well developed clear zones and ruffled borders were occasionally observed on the predentine surface, and they excavated resorption lacunae in the predentine. These results suggest that in vivo the multinucleation appears not to be an indispensable phenomenon for the membrane specialization of odontoclasts as reported by Prallet ²¹⁸.

Studies by Brudvik saw that the resorption process of teeth under movement was associated with remodelling of the PDL, more specifically the removal of hyalinized necrotic tissue²¹⁹. In TEM pilot studies multi-nucleated cells were observed during the process of removal of hyalinized tissue in orthodontic compression zones²²⁰. Studies confirm that multinucleated odontoclasts-like cells play important role in resorption of the root surface^{4, 221, 222}. Studies have suggested that mono-nucleated macrophage-like cells have ability to resorb mineralised dentine²²³. Resorption of the mineralised cementum has been reported to be initiated by mono-nucleated non-clast cells^{211, 224}. Light microscope study of the process of root resorption occurring beneath the hyalinized zone, indicated that multi-nucleated cells that were stained by tartrate

resistant acid phosphatase (TRAP) played an important role in the removal of necrotic tissue of the periodontal membrane and that similar cells were engaged in the removal of surface layers of cementum.

Macrophage-like, as well as the multi-nucleated cells without ruffled border are involved in the removal of the necrotic PDL and also capable of removing root cementum.¹³⁰ It was proposed that these cells resembling multinucleated giant cells, removed necrotic periodontal membrane (PM) tissue occurred adjacent to those parts of the cementum that had been reduced in thickness and otherwise seemed contaminated by the decay of necrotic tissue. It was assumed that removal of cementum occurred extracellularly and it suggested that the root surface was covered by damaged necrotic tissue being further contaminated by the breakdown-process of the main PM. The multinucleated cells having greatly contributed to elimination of the necrotic soft issue of the hyalinized zone perceived the unmineralized precementum as another necrotic and damaged tissue that should be removed. Brudvik then hypothesized that the multinucleated cells that resorbed the root surface were derived from the monocyte and macrophages that invaded the PM tissue. The question then remains whether these multinucleated cells are precursors to odontoclast involved in progressive resorption of cemental tissue.

3.8.2 How does the odontoclast resorb hard tissue

The compartment is acidified by the production of protons^{181, 225}. The acid etching of dental tissues by osteoclasts *in vitro* and odontoclasts *in vivo* show similar appearances. It has been suggested that the ruffled border zone is in effect an extracellular phagolysosome responsible for the digestion of the organic matrix of the

tissue being resorbed ²²⁵. Others refute this, claiming that the only action of the resorptive cells is demineralisation ²²⁶.

Recently Cathepsin, one of the lysosomal lytic enzymes that degrades bone and collagen together with cathepsin L ²²⁷ or is an activator of collagenase has been localised in the periodontal ligament during tooth movement. Cathepsin B was secreted and involved in the degradation of collagen ²²⁸. Cathepsin B activity seen in osteoclasts suggested a role of Cathepsin B in the resorption of bone by means of intra and extracellular degradation of bone matrix proteins. Cathepsin B activity was also located in foreign body giant cells adjacent to degenerating tissue. Cathepsin B was also seen distributed in the lingual half of the periodontal ligament. It was hypothesized that the fibroblasts synthesized actively cathepsin B for intracellular collagen digestion and extracellular collagen degradation through the activation of a latent type of collagenase ²²⁹.

Matrix metalloproteinase (MMP) ²³⁰ and cathepsin K ²³¹ are important in bone resorption. MMP can cleave type I collagen most efficiently within the triple-helical body of the native conformation and is active at neutral pH, whereas cathepsin K degrades type I collagen in a similar manner but is active at low pH in the acidic microenvironment. MMP mRNA was detected in cementoblasts, cementocytes and fibroblastic cells adjacent to odontoclasts in root resorbing tissue as well as osteoblasts, osteocytes and fibroblastic cells adjacent to osteoclasts in bone-resorbing tissue induced by tooth movement ²³². Cathepsin K mRNA was selectively expressed in odontoclasts and osteoclasts. This data supported that tooth root resorption and bone resorption are similar with regard to collagenolytic enzyme production.

The role of MMP is unclear both in bone and tooth resorption. There is evidence that MMP -1 is produced not by osteoclasts but by osteoblasts ^{19, 233-236}. MMP-1 secreted

by osteoblasts may remove non-mineralised organic material from bone surfaces, and thus expose resorption-stimulating bone mineral to osteoclasts ²³⁷. However Okamura et al ²³⁸ described that bovine odontoclasts, in resorbing tissue of deciduous teeth, expressed MMP-1 RNA.

The structure of tooth root still differs from bone in that its fluorine content is much higher and it completely lacks a blood supply. Various studies in the analysis of root resorption looked at the resorptive activities during resorption of deciduous teeth ²³⁸, ²³⁹. The possibility exists that odontoclasts assume a different function in resorption of deciduous teeth and in response to tooth movement.

3.9 Similarities and differences between bone and root cementum

Biochemical studies have shown that cementum has a chemical composition similar to bone. To about equal parts per volume, cementum is composed of water, organic matrix and mineral. About 50% of the dry mass is inorganic and consists of hydroxyapatite crystals. The remaining organic matrix contains largely collagens and a lesser degree mainly glycoproteins and proteoglycans.

3.9.1 Organic matrix

3.9.1.1 Collagens

The organic matrix of cementum consists primarily of collagens. Like bone and periodontal ligament, the two typical fibril forming collagens type I and III are also found in cementum. Biochemical analysis of human cementum have revealed that approximately 90% of the organic matrix is type I collagen and a minor proportion of

approximately 5% accounts for type III collagen^{240, 241}. The procollagen molecules are secreted and aggregated extracellularly to form cross-striated collagen fibrils with the typical 67nm banding pattern.

3.9.1.2 Non collagenous protein

Cementum is rich in glycoconjugates which represent either glycolipids, glycoproteins or proteoglycans and harbours a variety of other proteins.

Like bone, the predominant noncollagenous proteins are bone sialoprotein and osteopontin. Both are phosphorylated and sulfated glycoproteins.²⁴²⁻²⁴⁵ They bind tightly to collagenous matrices and hydroxyapatite, participate in the mineralization process and reveal cell attachment properties through the tripeptide sequence Arg-Gly-Asp (RGD) that binds to integrins²⁴⁶. Their definite roles in the mineralization process are not known.

Osteonectin is another glycosylated protein found in the extracellular matrix of mineralised tissues. As shown in bone, a close relationship between osteonectin and collagen seems to exist in the mineralization process²⁴⁷⁻²⁵⁰.

The two glycoproteins fibronectin and tenascin are widely distributed. One function of fibronectin is to bind cells to components of the extracellular matrix. During tooth development fibronectin and tenascin are present in the basement membrane of Hertwig's root sheath at the time of odontoblast differentiation²⁵¹. Later in development, they are also found at the attachment site of the periodontal ligament to the cementum surface but not the cementum layer itself²⁵².

The proteoglycans which are widely distributed in mammalian tissues, consist of a core protein to which sulphated polysaccharides are covalently linked. Biochemical

analyses of extracts from human cementum have identified chondroitin sulfate, dermatan sulfate and hyaluronic acid as the glycosaminoglycan constituents of these proteoglycans²⁵³.

Osteocalcin, a very small protein found in abundance in extracellular matrices of bone, dentin and cementum, appears to be involved in the mineralization process²⁵⁴.

The enzyme alkaline phosphatase is believed to participate in cementum mineralization. In rat molars the enzyme is heterogeneously distributed in the periodontal ligament, with the highest activity being found adjacent to alveolar bone and cementum²⁵⁵.

3.9.1.3 Mineral component

Cementum is generally less mineralised than root dentin from the same teeth^{256, 257}.

Acellular extrinsic fiber cementum appears more highly mineralised than cellular intrinsic fibre cementum and cellular mixed stratified cementum²⁵⁸.

Rockert²⁵⁹ examined cementum of monkey teeth by quantitative X-ray microscopy and found that the concentration of Ca varied within a wide range from 0.1 to 0.83 mg Ca/mm³. This variation confirms that cementum is not a completely mineralised or homogeneously mineralised tissue.

Chemical analysis and physiochemical studies indicate that the mineral component is the same as in other calcified tissues that is hydroxyapatite, with small amounts of amorphous calcium phosphates present. Studies indicate that the crystallinity of the mineral component is lower in cementum than in other hard tissues²⁶⁰. The minute size of the mineral crystals compared with enamel results in a much larger specific surface area of the mineral component. As a consequence, cementum has a greater capacity for adsorption of fluoride and other greater capacity for adsorption of

fluoride and other elements over time but also more readily decalcifies in the presence of acidic conditions.

As in other hard tissues, the hydroxyapatite of cementum is not chemically pure but contains other elements that have been taken up from the tissue fluid during initial crystallization.

The Mg^{2+} content in cementum is about half that in dentin. The composition of cementum is more similar to bone than to dentine.^{245, 257, 261}

Cementum appears to have a high fluoride content compared with other hard tissues. Fluoride concentration in cementum shows a general increase with age and varies with the nutritional fluoride supply to the individual^{221, 262}.

Although dental cementum is usually referred to as a bone-like tissue, there are obvious differences in regard to vascularity, cellular components and rate of turnover. Bone tissue, including alveolar bone, participates actively in the metabolism of the body and constitutes a reservoir of calcium and other elements that the body can draw upon as needed. The cementum is largely excluded from such processes.

A variety of noncollagenous proteins are stored in the mineralised matrix of cementum. Whereas most of these extracellular proteins are typical matrix constituents of collagen based mineralised tissue, others appear specific for cementum. Among these is a cementum derived attachment protein that mediates the attachment of connective tissue cells²⁶³⁻²⁶⁵. Some data indicate that this protein is different from both bone sialoprotein and osteopontin. On the other hand, the possibility cannot be ruled out that this protein may represent a degradation product or a posttranslational variant of known proteins. Another protein believed to be specific for cementum is a cementum-derived growth factor²⁶⁶. During root resorption and surgical instrumentation, proteins exposed to the root surface and /or released from

cementum and dentin could possibly influence the initiation of the repair process by cell migration, division, attachment and differentiation.

The two opposing hard tissues, dental cementum and alveolar bone, are strikingly different in histological structure, physiological activity, biochemical composition, as well as in age and reactivity of their organic and inorganic components.

4 Orthodontic tooth movement: bone resorption and root resorption

Orthodontic tooth movement is made possible by extensive bone remodelling. Somehow during this phenomenon, the roots are more or less spared. However the precise reasons are unknown. Several theories have been offered to explain the relative resistance of roots to resorption:

1. the innermost cellular layer of the periodontal ligament provides protection to the tooth by providing cementoblast, osteoblasts, fibroblasts, endothelial and perivascular cells for repair ⁷⁵.
2. The cellular layer covering the tooth surface, the Sharpey's fibers at the root side, the superficial layer of unmineralized matrix (precementum and preentin), and elements of the organic matrix as important contributors to resistance to resorption ²²⁶.
3. The unmineralized tissues, osteoid, precementum and preentin are resistant to resorption and may initially prevent root resorption ²⁶⁷.
4. Elevated levels of matrix metalloproteinases in cementum compared to other tissues ²⁶⁸.

5. An “anti-invasion” component in the cementum layer²²³.

When orthodontic force is applied, there is usually an attack on adjacent bone with cemental repair occurring at a relative passive rate after force levels decline. Therefore, root remodelling is a constant feature in orthodontic tooth movement and permanent loss of root structure will occur only if repair does not replace the initial resorbed cementum. Animal studies have indicated that root resorption tends to increase with the use of strong forces, human clinical studies suggest that the amount of force does not increase the risk of root resorption. This discrepancy could be explained not only by the magnitude of the force but more probably by variations in metabolic response amongst species or individuals. It was also shown that there was great variability between individuals in their response to multitude of factors (discussed above section 2). This indicated that the variation may be attributed also to chemical and or hormonal influences.

4.1 Controlling factors of resorption in dental tissue

4.1.1 Expression of RANKL and OPG in dental tissue

The first association of the importance of RANKL and OPG in the dental environment was seen when Kong et al¹³⁸ showed that mice with a disrupted RANKL gene show severe osteopetrosis and a defect in tooth eruption, and completely lack osteoclasts as a result of an inability of osteoblasts to support osteoclastogenesis. Rani and MacDougall in 2000²⁵ using odontoblast and pulp cell lines were able to detect a constitutive expression of OPG, RANKL and M-CSF in both cell types. In vivo, RANKL and OPG were localized to odontoblasts, ameloblasts and pulp cells in

developing mouse teeth by immunohistochemistry. Using co-culture methods Kanzaki et al ²⁶ confirmed that PDL cells also required cell to cell contact for osteoclastogenesis.

Studies looking at physiological tooth eruption indicate that OPG but not RANKL is expressed in the dental follicle of the rat mandibular molars ²⁹. However the gene expression of OPG is reduced in the first mandibular molar of the rat at day 3 post-natally, and in vitro expression is reduced when the dental follicle cells are incubated with either CSF-1 or PTHrP. Thus it has been postulated that CSF-1 and PTHrP, seen in the follicle and stellate reticulum, respectively may be responsible for the reduced gene expression of OPG ²⁹. The inhibition of gene expression of OPG at that time would allow for osteoclast formation, resulting in the maximal number of osteoclasts seen. RANKL is present in the alveolar bone and thus, the cell to cell signalling could occur to promote osteoclast formation ²⁹.

Cells derived from deciduous teeth showed down regulation of OPG after the application of 1α 25 (OH) $_2$ and dexamethasone. While RANKL mRNA was up-regulated by the same treatment³¹. There was an indication that PDL cells derived from deciduous teeth synthesized both RANKL and OPG and therefore could regulate the differentiation of osteoclasts.

Studies on human deciduous teeth have indicated similar expression of RANKL. Lossdorfer et al ³⁰ using immunohistochemical methods detected granular cytoplasmic RANKL immunoreactivity in odontoblasts, pulp fibroblasts, periodontal ligament fibroblasts and in single odontoclasts. They suggested odontoclasts therefore had an autocrine/paracrine role. This was supported by Myers et al ³² who demonstrated the occurrence of RANKL-mRNA in 2 of 8 microisolated rat osteoclast samples and 1 or 4 human osteoclast samples. RANK positive cells were identified as

multinucleated odontoclasts localized near the dentine surface in resorption lacunae or as mononucleated precursors. Expression intensities varied and it was assumed that cells were in various phases of activity or that different cell types express different levels of the protein. While also not all odontoclasts expressed either RANKL or RANK.

4.2 Regulation by RANKL and OPG during tooth movement

Shiotani et al ²⁶⁹ showed expression of RANKL protein in osteoblasts, osteocytes, fibroblasts and osteoclasts in resorption lacunae during orthodontic force application using immunohistochemistry, however they did not detail the number of animals used in the study. They concluded from their study that RANKL expressed in the cisternae of PDL cells, it was thought to reflect its biosynthesis.

Cell to cell interaction between osteoblasts/stromal cells and osteoclasts had been shown to be essential for osteoclast formation ¹⁷, they suggested that the PDL osteoblast/stromal cells were involved in supporting osteoclast differentiation in the periodontal tissues. They found RANKL expression in osteoclasts in the ruffled border membranes and clear zone cytoplasm, while Kartsogiannis et al ¹⁴⁹ reported that light microscopic RANKL expression was higher in osteoclasts located in resorption lacunae than in immature osteoclast precursors. RANKL was predominantly detected in osteoclasts and therefore was thought to be produced in the osteoclasts rather than by osteoclastic incorporation of soluble RANKL proteins which are also secreted by stromal cells in a paracrine manner. The demonstration of RANKL localization along the ruffled border membranes is quite similar to that of H⁺-ATPase ^{8, 9, 270, 271}.

RANKL expression was increased equally after force application in both OPG deficient and normal mice, however expression of RANKL was detected at day 0 in OPG deficient mice as compared with day 2 of wild-type mice. OPG deficient mice had severe resorption of alveolar bone. RANKL and OPG were thought to have a significant role in regulating balanced resorption²⁷².

There is limited information regarding the significance of controlling molecules that regulate the cellular process of the amount of root and/bone resorption that occurs during orthodontic force application. RANKL/OPG ratio have been shown to increase with increased bone resorptive activity in bone metabolic diseases²⁷³ and other conditions including giant cell tumor of bone and adjuvant arthritis³³. A recent study of the pathological processes of periodontal bone destruction during active periodontal disease showed that RANKL mRNA was increased in periodontitis at the advance stage. In contrast, the expression of OPG in periodontitis was decreased in both advanced and moderate stages. In situ hybridization showed that RANKL mRNA was expressed in both inflammatory cells and epithelial cells of gingival tissue affected by periodontitis²⁷⁴.

Therefore it is clear that much information has yet to be gained in regards to further increase our understanding of cellular signalling between dental cells of odontoblast/clasts, cementocytes, periodontal ligament cells and osteoblast/clasts during tooth movement and root resorption.

5 General Aims

- To study some cellular and molecular activities associated with root resorption using a rat tooth model.
- To identify and analyse expression levels/ratios of OPG and RANKL in normal and in areas of root resorption subsequent to the application of heavy orthodontic forces.
- To analyse these levels using extractive RNA methods and to semi quantify levels of OPG and RANKL.
- In general to better understand the biology of tooth movement and root resorption and its correlation with other bone remodelling processes, which could allow better clinical treatment of patients during appliance applications.

6 Literature review of specific methods

6.1 Animal model

The laboratory or Norway, rat (*Rattus norvegicus*) is one of the most commonly used experimental animals, offering as it does the best “functionally” characterized mammalian model system. The rat serves as a model organism for the analysis of a number of important biomedical traits, such as cardiovascular diseases, metabolic disorders, neurological disorders, neurobehavioral studies, organ transplantation, autoimmune diseases, cancer susceptibility and renal diseases. It offers a number of unique advantages for modelling human diseases, developing new therapeutic agents, and in studying responses to environmental factors. The size of the rat, in contrast to other commonly used experimental animal, makes it ideal for certain physiological manipulations. In addition, the recent development of genetic and genomic tools for the rat now provides an unprecedented opportunity to take advantage of the rich and robust history of experimental studies utilizing this species to study human disease. The wealth of past and recent information available on the rat and makes it a versatile model to use.

6.1.1 Rat oral anatomy

The upper lip is cleaved by a deep hairless groove, the philtrum which forms a ventral continuation of the “Nasenspiegel” phinarium. The dorsal wall of the oral cavity is formed by the hard palate, equipped with prominent palatal ridges, and the soft palate containing a thick layer of mucous glands. The tongue is grooved by the

median sulcus in the rostral third. At the transition from the middle to the caudal third, there is a distinct dorsal prominence.

The rat has 16 teeth, an incisor and three molars on each side of the jaws. There is a diastema between the incisors. The skin folds behind the incisors can close the mouth, preventing the swallowing of indigestible material ²⁷⁵.

The incisors erupt about the 10th day postnatally, the molars on the 19th and 22nd and 35th to 40th day for first, second and third molars respectively. By 6 weeks the entire set is in use ²⁷⁶.

The shape of the incisors, with almost geometric precision, is that of a segment of a spiral. The dentine in the incisor tooth increases in thickness toward the tip. It is covered on the labial surface by a reddish enamel layer, on the other sides by a layer of cementum. The colour of the enamel is due to its content of an iron-containing pigment. A sharp cutting edge results from the acute angle formed between the masticatory surface and the enamel plate. The normal wear of the masticatory surface and lower incisors is assured by the mobility of the temporomandibular joint in the sagittal plane. The tips of the lower incisors can be brought into a position either before or behind the tips of the upper incisors ²⁷⁶.

The apical foramen remains open throughout life; thus continuous growth of the tooth is possible. For enamel and dentin, a daily addition of 16µm has been measured. Fatani and Raja ²⁷⁷ found a daily growth of lower incisors of 15µm in adult rats, while in immature rats it is up to 0.1mm per day.

The periodontal ligament consists of two layers of fiber bundles ²⁷⁸. Blood supply of the periodontium shows a special pattern along the labial surface. Blood vessels arranged in three layers provide the material for enamel formation ²⁷⁹. The dental

pulp in the lower incisor is innervated by small numbers of loosely distributed thin unmyelinated axons. In close association they run through almost the entire length of the pulp, ending close to its incisal end. As there is no evidence of autonomic function they were suggested to be mainly sensory²⁸⁰. In the upper incisor the pulp contained myelinated and unmyelinated nerve fibres²⁸¹.

Incisors and molars are separated by a large interdental space. Depending upon age this varies from 12-20 mm in the upper jaw and 10-12mm in the lower jaw.

The row of three tightly apposed molar teeth of each quadrant increases in both length and width by about 10% in older animals²⁸². The size of the molars decreases from the first to the third. The masticatory surfaces contain transversely oriented enamel folds and tubercles. Up to nine tubercles can be counted on a molar in young animals²⁸³. Dependent on wear, three main transverse folds can usually be counted in the adult. First molar has five roots, second molar has four and third molar has three. Cementum covers one third to one half of the roots. The enamel folds are free of cementum. Within the extensively innervated pulp the nerves either end freely below the odontoblast layer or form gap junction endings on the odontoblast cell body or along its dentinal process²⁸⁴.

Associated with migration of molar teeth at a rate of 6.7µm per day Vignery and Baron²⁸⁵ describe rapid remodelling of alveolar bone tissue throughout life with a span of about 6 days for the remodelling cycle.

6.2 Analysis of gene expression

6.2.1 Why study gene expression

All cell and tissue functions are ultimately governed by gene expression; therefore the reasons for electing to study the modulation of RNA as one parameter of cellular biochemistry may be quite diverse. Some of the goals of experimental design may be any of the following

1. Measurement of the steady-state abundance of cellular RNA transcripts. This is the most commonly studied parameter of gene expression. Both quantitative and qualitative profiles of a population of RNAs can then be generated by Northern analysis, nuclease protection assay, or polymerase chain reaction (PCR)
2. Measurement of the rate of transcription of gene sequences or pathway of processing of RNAs. This can be deduced in part by nuclear runoff assays in which labelled ribonucleotide precursors are incorporated into nascent transcripts in direct proportion to the abundance of each RNA being transcribed.
3. Mapping of RNA molecules including the 5' end, 3' end, and size and location of introns. This can be derived from partial digestion of the RNA molecules of interest by nuclease protection assay and/or by PCR.
4. Cell-free *in vitro* translation of purified messenger RNA (mRNA). The resulting polypeptide may be further characterized by immunoprecipitation and/or western analysis. This is usually helpful in identifying newly isolated genes.

5. Synthesis of complementary DNA (cDNA). Purified mRNA can serve as the template for the enzymatic conversion of these relatively unstable, single stranded molecules into much more stable, double-stranded cDNA molecules. Among the more common reasons for performing cDNA synthesis are the amplification of the sequence by PCR, often for some “quantitative purpose”. This is the principle approach taken in the current study. Cloning by direct ligation of the cDNA into a vector, the resulting cDNA library may be propagated for long term storage and analysis.

6.3 RNA Isolation

Protocols for the isolation of RNA begin with cellular lysis mediated by buffers that typically fall into one of two categories

1. those consisting of harsh chaotropic agents including one of guanidinium salts, sodium dodecyl sulphate (SDS), *N*-laurylarcosine, urea, phenol, or chloroform, which disrupt the plasma membrane and subcellular organelles and which simultaneously inactivate ribonucleases.
2. those that gently solubilize the plasma membrane while maintaining nuclear integrity, such as hypotonic Nonidet P-40 lysis buffers. Nuclei, other organelles and cellular debris are then removed from the lysate by differential centrifugation.

6.3.1 Isolation of RNA with Guanidinium buffers

RNA lysis buffers that contain guanidinium thiocyanate or guanidinium-HCl reproducibly yield very high-quality RNA samples. This is true because of the

extremely chaotropic nature that these chemicals exhibit; they are among the most effective protein denaturants ²⁸⁶⁻²⁸⁸. This attribute supports the inclusion of guanidinium as the method of choice when planning an RNA extraction from whole tissue samples. The efficiency of protein denaturation may be enhanced by the inclusion of β -mercaptoethanol, or dithiothreitol, these reducing agents act to break intramolecular protein disulfide bonds.

6.3.2 Other methods of RNA isolation

6.3.2.1 Gentle lysis buffers

Cellular lysis mediated by non-ionic, hypotonic buffer is not disruptive to most subcellular organelles. The inclusion of NP-40 ²⁸⁹ and $MgCl_2$ in the lysis buffer facilitates plasma membrane solubilization while maintaining nuclear integrity. Thus initial lysis of cells with NP-40 containing buffer precludes the mixing of nuclear and cytoplasmic RNA from the onset. In such protocols intact nuclei, large organelles and cellular debris are easily removed by differential centrifugation. The resulting cytosolic supernatant is rich in cytoplasmic RNA and proteins, which is easily removed by series of extractions with organic solvents such as phenol and chloroform. The obvious advantage of this isolation method is that RNA precipitated at the end of the procedure represents only cytoplasmic population.

A disadvantage of this approach is that lysis buffer alone is not sufficiently chaotropic to fully inhibit or destroy RNase activity.

6.3.2.2 Isolation of cytoplasmic RNA by hypotonic lysis with NP-40

This procedure is based on non-ionic, hypotonic solubilization of the plasma membrane while maintaining nuclear integrity. Cellular lysis is accomplished as quickly as possible, on ice, in the presence of RNase inhibitors. Following gentle cell lysis the subsequent phenol:chloroform extractions and alcohol precipitation of the RNA are among the most common preparative techniques. Although better quality RNA may be generated using guanidinium based lysis buffers, RNA prepared according to this method is adequate for most standard applications.

6.4 Determination of Nucleic Acid Concentrations

6.4.1 Spectrophotometric methods

The concentration and purity of DNA and RNA samples are determined readily by taking advantage of the ability of nucleic acids to absorb ultraviolet light, maximally at 260nm. Absorbance measurements at this wavelength permit the direct calculation of nucleic acid concentration in a sample:

Concentration of RNA $\mu\text{g/ml} = A_{260} \times \text{dilution} \times 40.0$.

Where

A_{260} = absorbance (in optical densities) at 260nm

Dilution = dilution factor (usually 200-500)

40.0 extinction coefficient of RNA

The ultraviolet (UV) measurement is best taken by diluting the sample in an acid washed quartz cuvette.

6.5 Gene expression analyses

Several techniques for the analysis of gene expression at the mRNA-level are available, such as

1. Northern blotting²⁹⁰
2. Differential display²⁹¹
3. Serial analysis of gene expression (SAGE)²⁹² and
4. Dotblot analysis²⁹³.
5. Microarray analysis²⁹⁴⁻²⁹⁶
6. Real time RT-PCR³⁰¹
7. In situ hybridization

6.5.1 Northern blotting

There are an enormous number of variations on the theme of Northern analysis since the original descriptions of the technique²⁹⁰. Northern analysis is the transfer or blotting of electrophoretically separated RNAs from a gel to a filter membrane for subsequent fixation and hybridization to a specific probe or probes. Because the RNA is transferred in exactly the same configuration as it was separated in the gel, hybridization signals generated by Northern analysis provide a qualitative and semi quantitative profile of the sample under investigation.

6.5.2 Differential display

The orchestration of gene expression at the transcriptional level and beyond is responsible for the differentiation of cells and tissues and ultimately for conferring the phenotype of the organism. It is often the goal to be able to determine which genes

are expressed in common among two or more different cell types and more importantly which genes are expressed differentially when comparing these cell populations.

Prior to PCR, several variations of the method known as subtraction hybridization²⁹⁷⁻²⁹⁹ were utilized as a means of subtracting away sequences shared by two populations of RNA/cDNA.

Most investigators attempt to take advantage of efficiency and sensitivity of PCR. The strategy involves coupling standard methods for cDNA synthesis to PCR-based amplification using large combinations of short primers, for the purpose of evaluating two or more experimental populations in a side by side comparison by polyacrylamide gel electrophoresis.

6.5.3 Serial analysis of gene expression (SAGE)

SAGE uses automated DNA sequencing to efficiently count large numbers of mRNA transcripts from a small population of cells. SAGE increases the number of genes that can be counted per sequencing reaction, compared to cDNA library sequencing, by minimizing the portion of the transcript sequenced. The method works by cloning and sequencing a 10-base pair (bp) portion of the cDNA at a defined position near the 3' end of the transcript. This 10-bp portion, normally next to the last *Nla*III restriction site, is known as the transcript's 'tag'. SAGE tags are ligated and cloned end-to-end in a sequencing vector, allowing the 'serial' analysis of multiple transcripts. The number of times a particular tag is observed in a tag population made from one mRNA sample (SAGE library) is used to determine transcript abundance. The SAGE transcript profile from various types of cells can be archived on a computer database and

electronically compared to find statistically significant differences in gene expression between cell types.

6.5.4 Dot blot

The dot blot analysis allows the investigator to make quick statements about the biochemical composition of a sample. Dot blots permit a rapid detection of the relative amount of a particular RNA sequence. Salient information can be obtained without electrophoresing the sample or performing any sophisticated enzymatic manipulations.

6.5.5 Microarray analysis

The study of gene expression by DNA microarray technology (reviewed in: Watson et al., 1998; Duggan et al., 1999; Graves, 1999)²⁹⁴⁻²⁹⁶, which is still in development, is based on hybridization of mRNA to a high-density array of immobilized target sequences, each corresponding to a specific gene. Sample mRNAs are labelled as a complex mixture, usually by incorporation of a fluorescent nucleotide by oligo(dT)-primed reverse transcription. The labelled pool of sample mRNAs is subsequently hybridised to the array, where each messenger will quantitatively hybridise to its complementary target sequence. After washing, the fluorescence at each spot on the array is a quantitative measure corresponding to the expression level of the particular gene. The use of two differently labelled mRNA samples allows quantitative comparison of gene expression in both samples. The major advance of DNA microarray technology, as compared to conventional techniques, results from the small size of the array, which allows for a higher sensitivity, enables the parallel screening of larger numbers of genes and provides the opportunity to use smaller

amounts of starting material. Mainly, the introduction of fluorescent probes has made miniaturization of arrays possible ³⁰⁰. The scale of gene expression analysis is not only extended by the simultaneous analysis of large numbers of genes, but also because microarrays can be produced in series facilitating comparative analysis of a large number of samples.

6.5.6 Real time RT-PCR ³⁰¹

Real-time PCR is based on the detection and quantification of a fluorescent reporter. The signal increases in direct proportion to the amount of PCR product formed in the reaction. By recording the amount of fluorescence emission at each cycle it is possible to monitor the PCR reaction online during the exponential phase, in which the first significant increase in the amount of PCR product correlates with the initial amount of template. Quantification is based on the threshold cycle, defined as the cycle number at which fluorescence and, thus, the PCR product become clearly detectable above background noise. The threshold cycle value can then be translated into a quantitative result by generating a standard curve. The standard curve for a certain gene is compiled by plotting individual threshold cycle values of a series of dilutions against the logarithm of the corresponding volumes. An equivalent calculation, as for the specific genes of interest, should be done for a housekeeping gene, for example {beta}-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or ribosomal protein S9, to normalize for differences in the amount of total RNA/cDNA starting material.

6.5.7 In situ hybridisation

In situ hybridisation (ISH) is a technique for the localisation of specific nucleic acid sequences within individual cells. It is based on the complementary binding of a nucleotide probe to a specific target sequences of DNA or RNA in the cell. This may be endogenous DNA, mRNA, viral sequences, or bacterial sequences.

ISH was first described in 1969^{302, 303}, and has now gained widespread acceptance and utility. It has many applications in basic research and in diagnostic pathology. ISH provides a high degree of spatial resolution that permits the localisation of genes to chromosome³⁰⁴ the identification of translocations and other chromosomal abnormalities^{305, 306}, and the study of gene expression at the cellular and subcellular levels^{307, 308}. It has also been used extensively for the identification of viruses and other micro-organisms³⁰⁹.

6.6 PCR

The polymerase chain reaction (PCR) is a powerful tool for the amplification of trace amounts of nucleic acids and has rapidly become an essential analytical tool for virtually all aspects of biological research in experimental biology and medicine. Because the application of this technique provides unprecedented sensitivity, it has facilitated the development of a variety of nucleic acid-based systems for diagnostic purposes, such as the detection of viral, or bacterial pathogens as well as genetic disorders, cancers and forensic analysis³¹⁰.

PCR uses the basic elements of the natural DNA replication processes as well as the basic components of cellular DNA replication where DNA is unwound and each strand of the parent molecule is used as a template to produce a complementary

strand. The copying relies on the ability of nucleotides to base pair. In a simple buffer system a region of a template DNA molecule is copied by a DNA polymerase that uses deoxynucleotides as building blocks of the new strands. Sequence-specific oligonucleotide primers that bind to the template according to normal base-pairing rules define the region of the template to be copied. Strands are separated by heat, which causes denaturation. The oligonucleotide primers bind to complementary sequences on the templates and DNA polymerase then begins to add deoxynucleotides to 3'OH group of the primers, producing new duplex molecules. At the next heating step, these new double-stranded molecules are once again denatured and each single strand provides a primer binding site and acts as a template for further DNA synthesis³¹¹.

6.6.1 Theoretical framework of PCR

The PCR educt (target of interest prior to amplification) is amplified during the PCR procedure in an exponential manner. A mathematical description for the product accumulation within each cycle is

Equation 1

$$Y_n = Y_{n-1} \cdot (1 + E_v) \text{ with } 0 \leq E_v \leq 1$$

E_v represents the efficiency of the amplification, Y_n the number of molecules of the PCR product after cycle n , and Y_{n-1} the number of molecules of the PCR product after cycle $n-1$. To calculate the number of molecules of the PCR product after a given number of cycles from the starting amount of PCR educt, this recursive equation has to be solved. Since E_v stays constant for a limited number of cycles during the exponential phase of the amplification reaction, this is possible within a particular

period. Therefore the accumulation of the PCR product can be approximately described by Equation 2

$$Y = X \cdot (1 + E_e)^n$$

Y represents the number of molecules of the PCR product, X the PCR educt molecules, n the number of cycles, and E_e the efficiency with a value between 0 and 1. Equation 2 is valid only for a restricted number of cycles, usually up to 20-30. then the amplification process slows down to constant amplification rates, and finally reaches a plateau where the target is not amplified anymore. The overall efficiency (E) of the amplification process is dependent on the primer/target hybridization, the relative amount of the reactants, especially the DNA polymerase/target quotient, and it may vary with the position of the sample in the thermocycler or the presence of coisolated DNA polymerase inhibitors in different samples. The number of cycles for which Equation 2 holds true is partly determined by the amount of PCR educt. Target strand reannealing and enzyme saturation events are lead to a decline of E_v .

6.7 Reverse transcriptase PCR

Analysis of gene expression requires accurate determination of mRNA levels. This is done by converting mRNA to complementary DNA (cDNA) and subsequently amplified.

Standard RT-PCR offers a rapid versatile and extremely sensitive way of analysing whether a target gene is being expressed and can provide some semi-quantitative information about expression levels.

RT-PCR is based on the ability of the enzyme reverse transcriptase, and RNA-dependent DNA polymerase, to generate a complementary strand of DNA using the

mRNA as a template. The reverse transcriptase reaction can be performed on either total cytoplasmic RNA or on purified mRNA.

DNA contamination must be avoided. Various ways of eliminating DNA contamination include:

1. Use of a control sample omitting the reverse transcriptase step.
2. Use of DNase digestion step, or
3. Designing primers that amplify across an intron, thus allowing simple identification of contaminating DNA that contain the intron. This will give rise to a longer product than the RNA transcript.

To copy the mRNA to cDNA, this is often done using an oligo-dT primer that can anneal to the 3'-poly A tail of eukaryotic mRNAs and allows reverse transcriptase to synthesize cDNA from each mRNA molecule. There are two common types of reverse transcriptase: avian myeloblastoma virus (AMV) and Moloney murine leukemia virus (M-MLV). AMV-RT has both 5'→3' primer-dependent polymerase activity with either RNA or DNA as template and a 3' →5' RNase activity that degrades the RNA portion of the RNA-DNA heteroduplex product of cDNA synthesis. The M-MLV-RT is essentially identical to the AMV enzyme but it can only use RNA as a template.

6.7.1 Quantification of PCR products

In contrast to simple positive/negative determination, inherent features of the amplification process may constrain the use of PCR in cases where an accurate quantitation of the input nucleic acids is required. Although the theoretical

relationship between the amount of starting template nucleic acid and the amount of PCR product can be demonstrated under ideal conditions, this does not always apply to most typical biological or clinical specimens. Various parameters are capable of interfering with exponential nature of in vitro amplification process during PCR-based quantification of nucleic acids. Even very small differences in the kinetic and efficiency of individual amplification steps will have a large effect on the amount of product.

Inherent factors that will lead to tube to tube or sample to sample variability are, for example, thermocycler-dependent temperature deviations, the presence of individual DNA polymerase inhibitors in the samples, pipeting variations or the abundance of the target sequence in the specimens of interest ^{312, 313}.

Various approaches have been developed in the past to circumvent these problems, but the extremely desirable goal of truly quantitative PCR has been difficult to perfect.

The quantitative capability has been shown in previous studies. This was demonstrated by comparing classical means of nucleic acids quantitation, such as Northern blot or slot blot analyses. Noonon et al reported that the results of PCU-based quantitation of multidrug resistance (MDR1) gene expression in MDRKB cell lines show a good correlation with results obtained by the traditional slot blot technology ³¹⁴. Similar results were obtained when comparing MDR1 gene expression by quantitative PCR and Northern blot methodology ³¹⁵. Quantitative PCR has been shown to correlate with in situ hybridization. This was demonstrated by Park and Mayo ³¹⁶, who studied the temporal expression and localization of the progesterone receptor mRNA in the rat ovary. Their results demonstrated that an equivalent

proportionality in the relative amount of RNA was obtained by both PCR and in situ hybridisation.

6.7.2 Methods of Quantitation

6.7.2.1 PCR based quantification with external standards

Serial dilution of a known amount of standard, often a plasmid, can be amplified in parallel with the samples of interest. Provided that a linear PCR product/PCR educt relation for the standard dilution series is observed, the relative amount of PCR educt for samples in the same PCR run can be deduced. A potential drawback to this procedure is the sensitivity of the PCR for small variations in the setup.

The quantification with this procedure must be done in the exponential phase, which is also dependent on the relative amount of the PCR. Rigorous analyses have to be performed to demonstrate that with increasing number of cycles, the results do not change.

6.7.2.2 Quantification with non competitive internal standards

Depending on the extraction procedure applied, nucleic acids isolated from cellular material usually contain a lot of nontarget DNA or RNA. The presence of cellular nucleic acids facilitates the coamplification of one of these cellular targets with the target of interest within the same PCR tube (multiplex PCR). This second cellular target shares neither the primer binding sites nor the region in between the target of interest. For DNA-PCR, almost any gene will do. Typical targets are pyruvate dehydrogenase³¹⁷, proenkephalin²⁸, or β -actin³¹⁸. For RNA-PCR, a cellular RNA has

to be selected that has an even level of transcription. JLA, β -actin, DHFR or GAPDH has been used³¹⁹.

The main advantage of this procedure is its simplicity and the fact that no profound molecular biology is needed. Replicates rule out tube-to-tube and to some extent sample-to sample variations, although individual inhibitors of the polymerase may be missed. On the other hand, this method bears some pitfalls that should be kept in mind. The efficiency of the reverse transcription for internal standard and the target of interest may vary and it may even vary dramatically for the same target³²⁰.

6.7.2.3 Competitive PCR

For competitive PCR, an internal standard has to be constructed that competes with the primary target for enzyme, nucleotides and primer molecules. The competitor bears the same primer binding region, but the sequence in between is modified in such a way that amplification products derived from the competitor and the target of interest can be differentiated, for example, by gel electrophoreses, enzyme-linked oligonucleotide sorbent assay, or HPLC. As long as the number of molecules of both PCR educts are equal, it is theoretically possible to use a competitor within a nested PCR assay³²¹.

For initial attempts, competitors were used that differ from the wild-type target only by a point mutation. In most cases, these point mutations are introduced in such a way that an additional restriction enzyme recognition site is created with the competitor nucleic acid^{322, 323}. Following restriction enzyme cleavage, the resulting products of competitor and primary target can be easily separated by electrophoresis on an agarose gel and quantitated by hybridisation with a labelled probe or with the help of a labelled PCR primer.

Although these competitors are showing a very high degree of similarity to the wild-type product, this procedure is difficult to regard as quantitative, due to the fact that the amplification products have to be diluted and that a second enzymatic step is necessary. In particular, if the amplification products of the competitor are not cut completely by the restriction enzyme, a false quantification results.

Developments in this technique have introduced deletions of a part of the wild-type sequence or insertions of foreign sequences are used for the *de novo* construction of competitors, which are analysed by gel electrophoresis³²³. There has been no general rules or strategies for construction of these modifications.

6.8 Detection and quantitative measurement of PCR products

To detect the presence and specificity of amplified DNA and to quantitate the amount of specific PCR products present in the reaction mixture, the amplification system has to be linked to an appropriate detection system. The amplification product have to be equipped with any kind of label that can be detected subsequently either in a direct or indirect way. For many years, the most commonly used methods for the detection of PCR- amplified DNA were based on radioactive labels. Due to the difficulty encountered in the handling of such radioactive isotopes, a variety of highly sensitive nonradioactive indicator systems have been developed. Suitable non- radioactive labels include helix-intercalating dyes, like ethidium bromide or bis-benzimide³²⁴, covalent bound dyes (eg fluorescein) or enzymes (eg. Horseradish peroxidase³²⁵ and alkaline phosphatase³²⁶ as well as distinct reporter molecules, such as digoxigenin or biotin.

Since the PCR is based on the oligonucleotide-primed synthesis of template-complementary DNA by the enzymatic action of a DNA polymerase, nonradioactive reporter molecules can be easily incorporated into amplification products either in the presence of labelled deoxyribonucleotide and/or labelled primer oligonucleotides present in the amplification mixture ³²⁷. Labelled deoxyribonucleotides are commercially available in the form of digoxigenin or biotin-dUTP. Primer oligonucleotides can be precisely labelled at the 5'-end during their chemical synthesis using digoxigenin-, biotin- or fluorescein-phosphoramidite components. Biofunctional conjugates, like anti digoxigenin antibody fragments or streptavidin , covalently linked to the enzymes horseradish peroxidase or alkaline phosphatase have been commonly used for detection of labelled PCR products in an ELISA type reaction.

6.9 Non sequence-specific detection systems

PCR applications already optimized with regard to the buffer $MgCl_2$ condition, temperature profile and other parameters, leading to well defined amplification products without the formation of any by-products that are different in size. Under suitable condition, the relative amount of amplification products is strictly dependent on the amount of starting material present in the amplification mixture. Therefore, quantification of the PCR products by physical or enzymatic means is sufficient for a rough determination of the amount of PCR product.

Different detection systems include:

1. Gel systems

Most simple are agarose or polyacrylamide gel electrophoresis and subsequent quantitative detection of ethidium bromide-stained

amplification products using gel scanners or suitable computer assisted video equipment. More recent use of automated DNA sequencers for the quantification of fluorescence-labelled nucleic acids. With the use of these instruments, gel associated lack of sequence specificity can be overcome by accurate size determination in the base pair range and ultimate detection sensitivities in the femtomole range of individual dye labelled amplification products.

2. HPLC

Direct HPLC of PCR products using for example, a 2.5- μm nonporous polymer-based an ion-exchange column, a 12-25min gradient cycle time and UV absorbance detection have been shown to meet the analytical criteria for practical PCR product quantitation³²⁸.

3. Solid phase Assays

The most commonly used and convenient solid-phase plastic support medium for bioanalytical assay is the 96-well microtiter plate. These plates can be some what automated.

4. Transcription-mediated detection

Quantitative measurement of specific mRNA species can be achieved by a combination of RT-PCR and a subsequent in vitro transcription reaction. In the course of this method, a T7 RNA polymerase promoter sequence is incorporated during the PCR reaction at the 5'-end of the amplification products and following the amplification reaction, an invitro transcription reaction is carried out in the presence of labelled ribonucleotides.

6.10 Sequence specific detection systems

Although the theory of PCR is straightforward, the primers are frequently annealing to nontarget sequences, especially in complex template mixtures, and this so-called mispriming, significantly lowers the purity of the amplified target portion in the final product. Therefore a probe-based methods are used.

Nonisotopic colorimetric systems are now currently used as opposed to previous isotopic ones. Reporter molecules such as digoxigenin, biotin, or distinct enzymes, or dye molecules capable of emitting light (chemiluminescence) are used. Probes are mainly bound to wells of microtiter plates since this format has certain advantages for reproducible results and automation.

Examples of sequence specific detection systems are:

1. Dot blot procedures

Here the amplified target is denatured, immobilized on a nitrocellulose or nylon membrane surface and then hybridized with an appropriate labelled DNA probe.

2. Solid phase capture

- a. Immobilized capture probe

Oligonucleotides representing a characteristic part of the amplified sequence, called capture probes, are attached either covalently or via biotin:streptavidin linkage to a solid phase, and labelled PCR products are hybridized using stringent conditions.

- b. Immobilized amplification product

Biotin labelled PCR products are attached to a streptavidin-coated solid phase and subsequently hybridized with a labelled

probe complementary to internal sequences of specific amplification product. Another possibility is the covalent binding of amplified amplification products to carboxylated wells of microtiter plates³²⁹.

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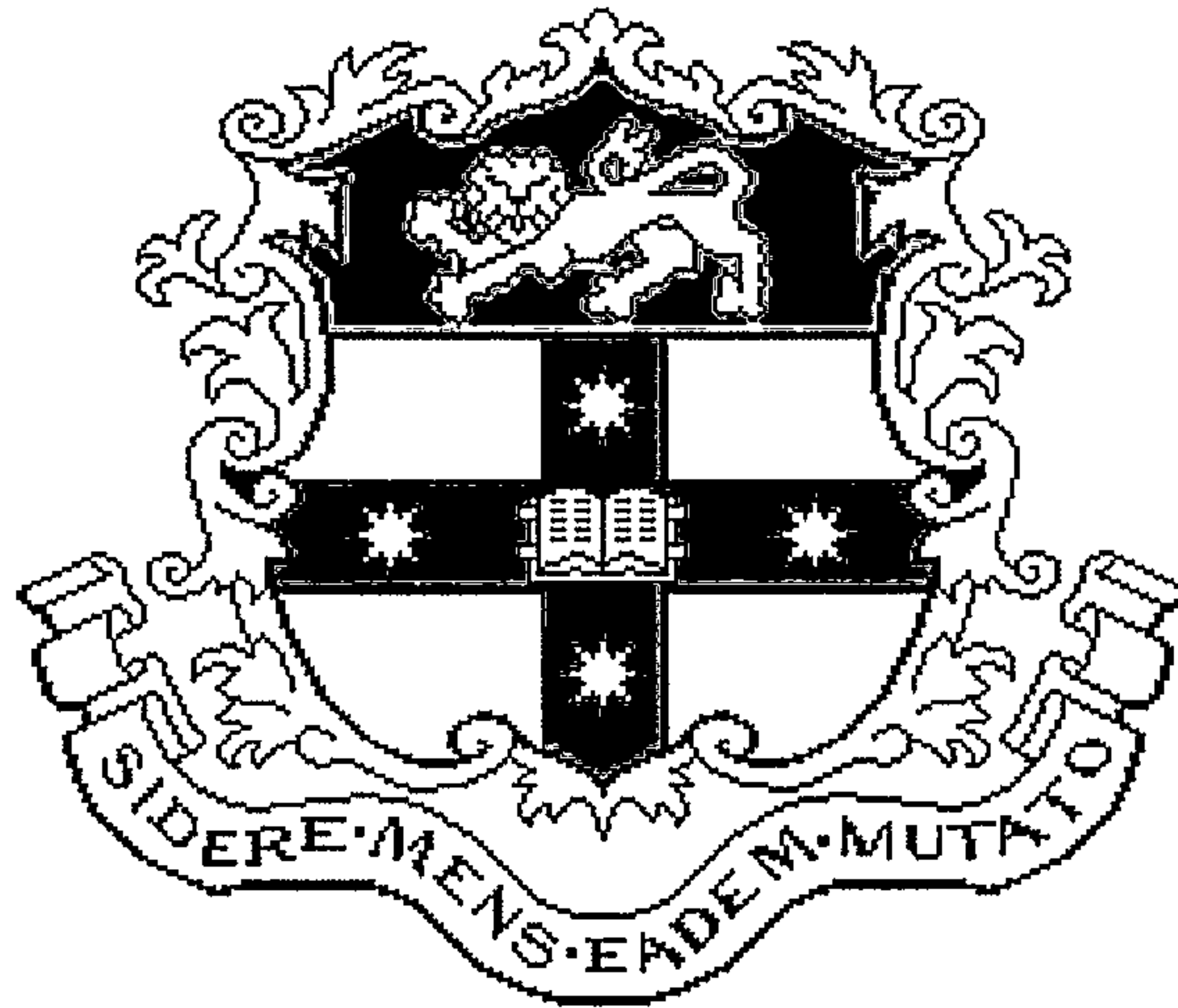
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Manuscript



Expression of mRNA for Osteoprotegerin and Receptor Activator of Nuclear Factor Kappa β Ligand (RANKL) during root resorption induced by the application of heavy orthodontic forces on rat molars

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Abstract

Receptor activator of nuclear factor kappa β ligand (RANKL) activates osteoclast differentiation while this activity is blocked by osteoprotegrin (OPG), so that the relative expression of these two proteins may contribute to bone and root resorption during orthodontic tooth movement. Here we describe experiments investigating RANKL and OPG mRNA expression in rats subjected to orthodontic forces. Fixed Sentaloy closed coil springs capable of delivering approximately 100 g of force were applied for mesial movement of the mandibular left first molar in 9 separate 7 week old male Wistar rats, using the right mandibular molars as internal controls for each animal. After 14 days, animals were sacrificed and tissues from 2 rats examined by paraffin histology while high quality mRNA was extracted from 4 mm widths of the mesial bony tissues in the remaining animals. Paraffin sections revealed osteoclastic resorption of roots on the mesial surfaces of teeth subjected to orthodontic forces. The integrity of mRNA was confirmed by RT-PCR for the housekeeping gene GAPDH, while that of primers specific for OPG and RANKL was determined by RT-PCR for these genes in material isolated from the UM106 rat cell line known to express both of these proteins. Densitometric analysis of RT-PCR OPG product revealed an increase in background levels of OPG mRNA in bony tissues subjected to orthodontic forces in all animals studied ($p < 0.05$). In contrast, low levels of mRNA for RANKL were detected in 5 animals only and only in association with orthodontic forces. Data are consistent with changes in levels of OPG and RANKL in tissues subjected to orthodontic forces and experiencing root resorption.

Keywords: RANKL, OPG, rat, semiquantitative PCR, root resorption

Introduction

Osteoclasts play a central role in bone and root resorption¹ so that molecular characterization of osteoclast differentiation is of potential clinical importance. Of particular interest for the current study, are three recently identified members of the tumour necrosis protein family: receptor activator of nuclear factor kappa B (RANK); RANK ligand (RANKL)(also known as Osteoclast differentiation factor, Osteoprotegerin Ligand, and Tumor necrosis factor-related activation-induced cytokine) and osteoprotegerin (OPG) also described as osteoclastogenesis-inhibitory factor.² RANK is a membrane bound receptor, first identified in a dendritic cell cDNA library³ and known to be expressed in numerous human tissues including skeletal muscle, thymus, liver, trabecular bone, adrenal glands, lung, brain and kidney.³ RANK is activated by binding either soluble or membrane bound RANKL,⁴ while this interaction is blocked by OPG, a secreted protein. In this way, the relative proportion of RANKL and OPG determines the activation or not of RANK, with increasing RANKL / OPG ratios associated with RANK dependent cellular activation.⁵ Osteoblasts and stromal cells are capable of secreting both RANKL and OPG,⁴ while RANK activation in macrophages results in osteoclastic differentiation and activation.⁴ Because of this, it has been proposed that altered RANKL and OPG expression by osteoblasts and or stromal cells may play a key role in root resorption during orthodontic tooth movement.²

There is some evidence of a role for RANKL and OPG in osteoclast activation during orthodontic tooth movement. In particular, Shiotani et al⁶ report the presence of RANKL protein in osteoblasts, osteocytes, fibroblasts and osteoclasts during application

of orthodontic forces. There is no difference in RANKL expression between OPG deficient and normal mice after application of orthodontic forces, although there is severe alveolar bone resorption in OPG deficient animals, supporting the idea that the relative expression of RANKL and OPG contributes to osteoclast activity.⁷ Interestingly for the current study, RANKL/OPG ratios increase with increased bone resorption in both metabolic bone diseases⁵ and active periodontal disease.⁸

In the current study, we describe changes in expression of RANKL and OPG mRNA as detected by reverse transcriptase polymerase chain reaction (RT-PCR) during root resorption induced by application of the heavy orthodontic forces in rats.

Materials and Methods

Application of Orthodontic Devices and Tissue Harvesting

Twelve 7 week old male Wistar rats with an average body weight of 450g were used. Animals were anaesthetised with Ketamine (90 mg/kg body weight) and rompum (10mg/kg body weight) for application of orthodontic devices. In all cases, the method modified from that described by Kobayashi et al.⁹ was used to apply a mesial traction force to the left first mandibular molar. Briefly, ligature wire loops placed around the cervical region of the left first molar teeth were used to fix Sentalloy closed coil springs (GAC, Cat No: 10- 000-09), each delivering approximately 100g of force as confirmed by tension tests on an Autograph- AG-50NE. The springs were then fixed anteriorly using ligature wire loops placed around the incisal thirds of both lower incisors. Composite resin was then placed over ligature wire ends to prevent mucosal trauma (Fig. 1). Control tissues were from the right mandibular molars of animals receiving appliances. After 14 days of treatment, rats were sacrificed by carbon dioxide asphyxiation and

tissues harvested for analysis. Approximately 4 mm³ volumes of bony and root tissues were dissected from the mesial aspect of molars using a dissecting microscope and high speed fine diamond discs and burs. These tissues were then snap frozen in liquid nitrogen and stored at -70°C for later extraction of mRNA. Where tissues were intended for histological analysis, mandibles were removed and fixed in 10% neutral buffered formalin. Throughout the study, animals were fed ground laboratory chow and provided with water ad libitum although weight loss from 10% to 15% was noted upon sacrifice.

Histological confirmation of root resorption

Mandibles were first fixed with paraformaldehyde (4%) in phosphate buffered saline (PFA) and then decalcified in EDTA (10%) in diethylpyrocarbonate treated PFA at 4°C for 10 days. Block dissection of the mesial root tissues and bone for both right and left sides were performed, and tissues embedded in paraffin for the preparation of 4 µm sagittal sections. Sections were stained with haematoxylin and eosin for light microscopy.

RNA isolation

High quality total RNA was isolated using a method modified from that described by Thomas et al.¹⁰ Briefly bone and root tissues were homogenised under liquid nitrogen using a mortar and pestle. The resulting tissue suspensions in liquid nitrogen were then evaporated into disposable plastic petri dishes before addition of TRIZOL (Life Technologies, Vic. Australia Cat No. 15596), 3 ml/gm of tissue. Tissue was then further homogenised using a polytron (Brinkmann, Westbury New York, PT 6100) and the

resulting supernatants transferred to centrifuge tubes containing chloroform (600 μ l per gm of tissue) and shaken for 15 sec before allowing to stand at room temperature (RT) for 3 minutes. Samples were clarified by centrifugation at 12,000g at 4°C for 15 min. Upper aqueous phase material was transferred to new eppendorf tubes before addition of 1.5 ml/ gm tissue of isopropyl alcohol. Samples were then incubated for 10min at room temperature and centrifuged for 10 min at 12,000g at 4°C. Supernatants were then discarded on ice and ethanol (75%) was added to wash pellets by vortexing. Precipitated RNA was then re-pelleted by centrifugation at 7,500 g for 5 min at 4°C before discarding supernatants on ice and air drying RNA pellets for 5 min at RT. Extracted RNA was stored at -70°C.

Reverse transcription-polymerase chain reaction (RT-PCR)

First strand complementary DNA (cDNA) was synthesised from 2 μ g of total RNA from each sample collected, by reverse transcriptase (Super-script II, Bibco) using random hexamer primers. cDNA was amplified by polymerase chain reaction (PCR) using primers purchased from Sigma (St Louis, USA) specific for OPG, RANKL and the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table I) and Taq polymerase (Roche Diagnostics Basel, Switzerland) in a Corbett thermocycler (Australia). GAPDH was used to control for variations in mRNA levels between samples. PCR was performed for 31 cycles in the case of GAPDH and 40 cycles for OPG and RANKL all at 55°C annealing temperature. In all cases the primers (Table I) were mRNA specific in that the recognition sites of the upstream and down stream primers resided in separate exons or at intron/exon boundaries of the genomic sequence. To allow semiquantitation of PCR products, preliminary experiments were performed to ensure that the number of PCR cycles were within the exponential phase of the

amplification curve. Amplification products were resolved by electrophoresis on a 2% wt/vol agarose gel stained with ethidium bromide and images captured with Gene snap (Medos, Victoria, Australia) for quantitative analysis using Gene tools (Medos, Victoria, Australia). The amplified products corresponding to OPG and RANKL are represented as a ratio of the respective PCR product relative to GAPDH PCR product.

Results

Orthodontic Forces Induced Root Resorption

Examination of paraffin sections revealed resorption lacunae associated with giant cells on the mesial root surfaces of teeth subjected to orthodontic forces (Fig. 2). These resorption lacunae varied in extent, extending variably into the cementum only, or alternatively, penetrating through into dentine (Fig. 2). Some multinucleated cells, however, were not located within lacunae but were instead within the periodontal ligament tissues (Fig. 2). Also noted within the periodontal ligament, were foci of lymphocytic infiltration, presumed to reflect an inflammatory response to orthodontic force applied. This contrasted with the appearance of control teeth not subjected to orthodontic forces, in which no resorption lacunae or giant cells were seen. This confirmed that the experimental model system used was appropriate for study of molecular events during root resorption.

OPG mRNA was Expressed in Control Tissues and Increased in Amount Upon Application of Orthodontic Forces

Figure 3 shows a typical result for OPG RT-PCR in a single rat as compared with GAPDH RT-PCR. The high quality of the mRNA extracted was evidenced by the presence of bands specific for both gene products tested. OPG was present in all samples tested, although levels appeared higher in the tissues subjected to orthodontic forces as compared with control tissues. This was confirmed by densitometric analysis, in which the ratio of OPG relative to GAPDH was compared for both test and control tissues, with orthodontic forces increasing OPG product levels 1.4 to 7.2 fold above that seen in control sites in all animals studied ($p < 0.05$) (Table 2). All densitometric analysis are shown in Figure 4 A and B. While the quantitative evaluation of expression levels are demonstrated in Table 3. Analysis of quantity was done by assigning a baseline quantity to a standard amount of 0.5 μ g of nucleic acid that was loaded as a marker. All quantitative values are shown as a ratio of expression of quantity compared with standard. The ratio of quantity of expression of OPG versus GAPDH is also shown. It is observed as stated above that the ratio of OPG to GAPDH in test animals were higher than controls.

RANKL mRNA was Only Detected in the Presence of Orthodontic Forces

Figure 5 shows the result of RANKL RT-PCR for both test and control tissues of a single animal, while similar results were obtained for a further 5 animals. Product for this gene was comparatively sparse as compared with both GAPDH and OPG, while no RANKL product was detected in material from 2 out of 7 animals despite the presence of both OPG and GAPDH product from these samples. Importantly, RANKL RT-PCR product

was only ever seen in the presence of orthodontic forces, with no product ever being detected in control tissues.

RANKL expression levels were analysed using Gene snap (Medos,, Victoria, Australia) for quantitative analysis using Gene tools (Medos, Victoria, Australia). Expression levels of test was compared with control. It was observed that expression was not seen in an ethidium bromide stained gel. Quantitative evaluation showed decrease in expression compared with positive control cells (UMR 106 cells) however were barely detectable in rat sample tissue collected from control side. This is shown in Figure 6 A and B. The graphs of intensity peaks are demonstrated in Figure 7. Intensity values are shown in below each graph.

Discussion

The method used to induce root resorption was modified from that described Kobayashi et al.,⁹ while histological assessment of tissues in the current study confirmed that this approach induced root resorption upon application of orthodontic forces. Interestingly, the multinucleated giant cells observed were not exclusively located in root resorption lacunae, but were also present in the periodontal ligament tissue proper. This is consistent with the reported role of cells with identical morphology in removal of the hyaline zone induced by high levels of orthodontic force (Brudvik and Rygh ,1993, 1994),¹¹⁻¹⁴ while it has been proposed that these cells can be recruited to the tooth surface and consequently mediate root resorption upon differentiation to an osteoclast/odontoclast phenotype.^{15, 16}

The methodology used to extract and amplify mRNA for specific gene products was confirmed as appropriate by detection of specific GAPDH, OPG and RANKL RT-PCR

product not only from experimental samples, but also from the UMR106 rat cell line expressing each of these proteins.¹⁷ Although OPG mRNA was detected in all samples tested, levels of mRNA for this gene product increased upon application of orthodontic forces, consistent with a role of this protein in inflammatory processes.¹⁸⁻²⁰

RANKL mRNA levels, appeared however, to be significantly lower as compared with OPG, and were undetectable in some animals. Nonetheless, where RANKL was detected, it was only ever in association with orthodontic forces. Since it has been argued that the relative expression of RANKL and OPG may determine the development or otherwise of osteoclasts, it is possible that the expression of RANKL mRNA in some sites subjected to orthodontic forces may reflect a role for this protein in osteoclast differentiation.

Arguing against this, however, are the very low levels of RANKL RT-PCR product detected relative to its competitive inhibitor OPG, which would appear to have always quantitatively exceeded RANKL in this experimental system. It may be possible to reconcile the apparent absence of RANKL mRNA in some sites subjected to orthodontic forces with the proposed role of this protein in osteoclast differentiation,^{21, 22} if it is proposed that in some animals, most multinucleated cells were engaged in removal of hyaline material in the periodontal ligament with only some cells committed to full osteoclast differentiation. This would be consistent with the presence of multinucleated cells separate from the root surface but in the periodontal ligament reported here. Also, since OPG is known to be widely expressed during inflammation,¹⁸⁻²⁰ while cytokine activity is highly dependent upon local spatial relationships within tissues, it is likely that much of the OPG mRNA detected in the current study was spatially distant from the root surface and thus unable to influence osteoclast differentiation. Further investigations beyond the scope of the current study are indicated to determine the precise localization of OPG and RANKL expression in this experimental model system.

Conclusion

In conclusion, RANKL and OPG levels were seen to increase in the environment during root resorption with the application of heavy forces, and may play a significant role during root resorption processes following orthodontic tooth movement.

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Table 1. Primers used in RT-PCR for OPG, RANKL and GAPDH.

Gene sequence mRNA	Oligonucleotide sequence 5' to 3'	Sequence based on Accession No.	Product Size (Base Pairs)
Rat GAPDH	ACCACAGTCCATGCCATCAC TCCACCACCCTGTTGCTGTA	AB017801	454
Rat OPG	TCCTGGCACCTACCTAAAACAGCA CTACACTCTCGGCATTCACTTTGG	U94330	578
Rat RANKL	ACGCAGATTTGCAGGACTCGAC TTCGTGCTCCCTCCTTTCATC	AF019048	493

Primers for GAPDH, OPG and RANKL were designed to flank intron-exon boundaries, in order to ensure that neither genomic DNA or unspliced mRNA would contribute to PCR product detected.

Table 2. Relative expression of RT-PCR product for OPG normalized for GAPDH levels in tissues.subjected to orthodontic forces (Test) as well as in control sites (Control) from 7 separate rats as determined by quantitative densitometry of ethyidium bromide DNA electrophoretic gels.

OPG (Densitometry Units) / GAPDH (Densitometry Units) (Relative Densitometry Units)			
Rat	Control	Test	Fold Change in the OPG / GAPDH ratio with Orthodontic Force
A	0.90	1.7	1.9
B	0.38	1.4	3.7
C	0.25	1	4
D	1.2	5	4
E	1.0	1.4	1.4
F	0.7	5	7.2
G	0.6	1.2	2.0

When OPG RT-PCR levels were quantitated by densitometry and divided by GAPDH levels as determined in parallel PCR reactions and identical gels, OPG RT-PCR product increased upon application of orthodontic forces ($p < 0.05$) in all animals studied, with the increase in band intensity ranging from 1.4 to 7.2 times that observed in control tissues.

Figure Legends

Figure 1 A. Schematic diagram of the orthodontic device applied to rat molars for induction of a mesial traction force.

Ligature wires placed around the cervical region of lower left first molar teeth as well as of lower left incisors were used to fix Sentalloy closed coil springs delivering approximately 100g of force of traction. Free ends of ligatures were covered with composite resin to avoid soft tissue laceration. The dashed rectangle indicates the hard tissues collected for molecular analysis.

Figure 1 B. Photograph of orthodontic device in situ applied to rat molar for induction of mesial traction force.

A Sentalloy closed coil spring (S) is attached to molar (M) and incisor (I) as described in Fig. 1A. The ligature ties are covered by composite (C). Left buccal mucosa is seen (B).

Figure 2. Paraffin sections of the mesial surface of a tooth subjected to orthodontic forces (A) and a control tooth (B).

The normal structure of the tooth and periodontal ligament was readily recognized, including pulp (P), dentine (D), cementum (C), the periodontal ligament (PL) and bone (Bn). Occasional large vessels (V) were noted entering the periodontal ligament from the adjacent alveolar bone in both control and test tissues. In teeth subjected to orthodontic forces, resorption lacunae were noted, often eroding through the cementum into the dentine (arrows) while a chronic inflammatory infiltrate and multinucleated giant cells (arrow heads) were also present, although occasionally not within lacunae (red arrows). These observations are consistent with the induction of root resorption by multinucleated giant cells in teeth subjected to orthodontic forces. (H&E, Bars = 100 μ m)

Figure 3. Ethydium bromide stained DNA electrophoretic gel of RT-PCR product for OPG (arrow) and GAPDH from tissue subjected to orthodontic forces (Test) as well as from the control site (Cont.) of a single rat.

The positions of base pair (BP) standards are indicated (Std.). RT- PCR product for OPG mRNA was detected in bony tissues both the presence and absence of orthodontic forces, but appeared to be significantly increased in amount in test as compared with control tissues, while GAPDH levels were comparable for both sites. Similar observations were made for 6 other separate animals.

Figure 4 GelDoc intensity peaks for expression of GAPDH and OPG in test samples of rat mandible

Each graph shows the intensity peak as demonstrated by GeneTools software.

Each table below the graph identifies the Rat from which the sample was taken and indicates the relative quantity of expression of OPG to GAPDH

Height indicates the base pair size of product analysed.

Raw volume is the volume assigned by the program as the intensity value of the area of band detected

Figure 5. Ethydium bromide stained DNA electrophoretic gel of RT-PCR product for RANKL (arrow) from tissue subjected to orthodontic forces (Test) as well as from the control site (Cont.) of a single rat. The positions of base pair (BP) standards are indicated (Std.). RT-PCR product for RANKL mRNA was only detected in test and never in control tissues. Similar observations were made for 4 other separate animals, while no RANKL RT-PCR product was detected from 2 animals.

Figure 6A and B RANKL amplification form two Rat mandibles

Each figure shows 3 lanes

Lane 1: molecular weight marker, lane 2 is amplification signal from RNA collected from control side of rat mandible, lane 3 is amplification signal from RNA collected from test side of rat mandible

Figure 7 RANKL expression levels as analysed by Gene Tools

Graphs generated from Gene Tools program. Each graph shows intensity signals from 4 different rats in relation to a standard.

Each graph shows the intensity peak as demonstrated by GeneTools software.

Each table below the graph identifies the Rat from which the sample was taken and indicates the relative quantity of expression of RANK comparing test and control side of each animal

Height indicates the base pair size of product analysed.

Raw volume is the volume assigned by the program as the intensity value of the area of band detected

Graphs 1-3 show results from Rat 1

Graphs 4-8 show results from Rat 2 and 3

Graph 9-11 show results from Rat 4

Figures

Figure 1

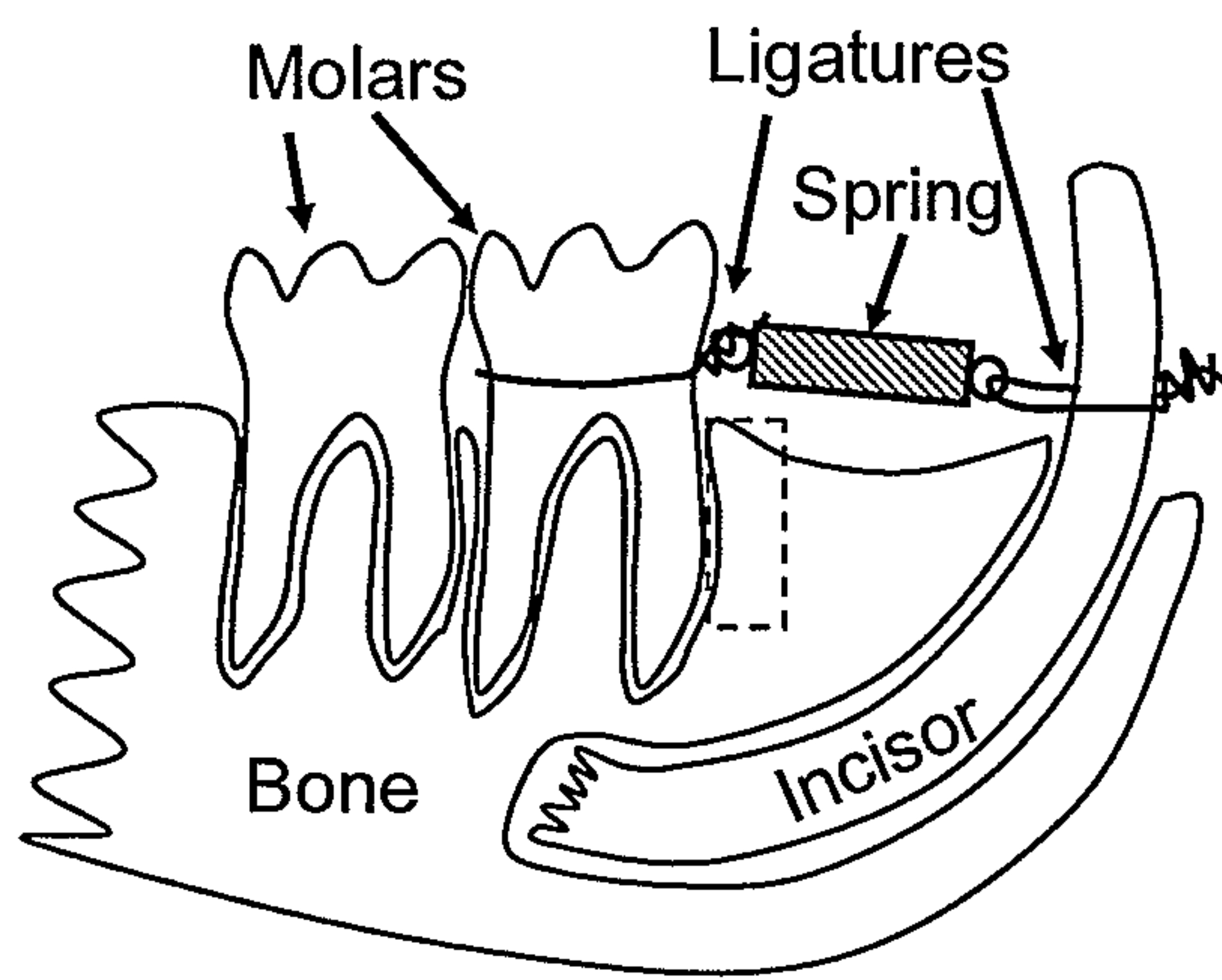


Figure 2



Figure 3



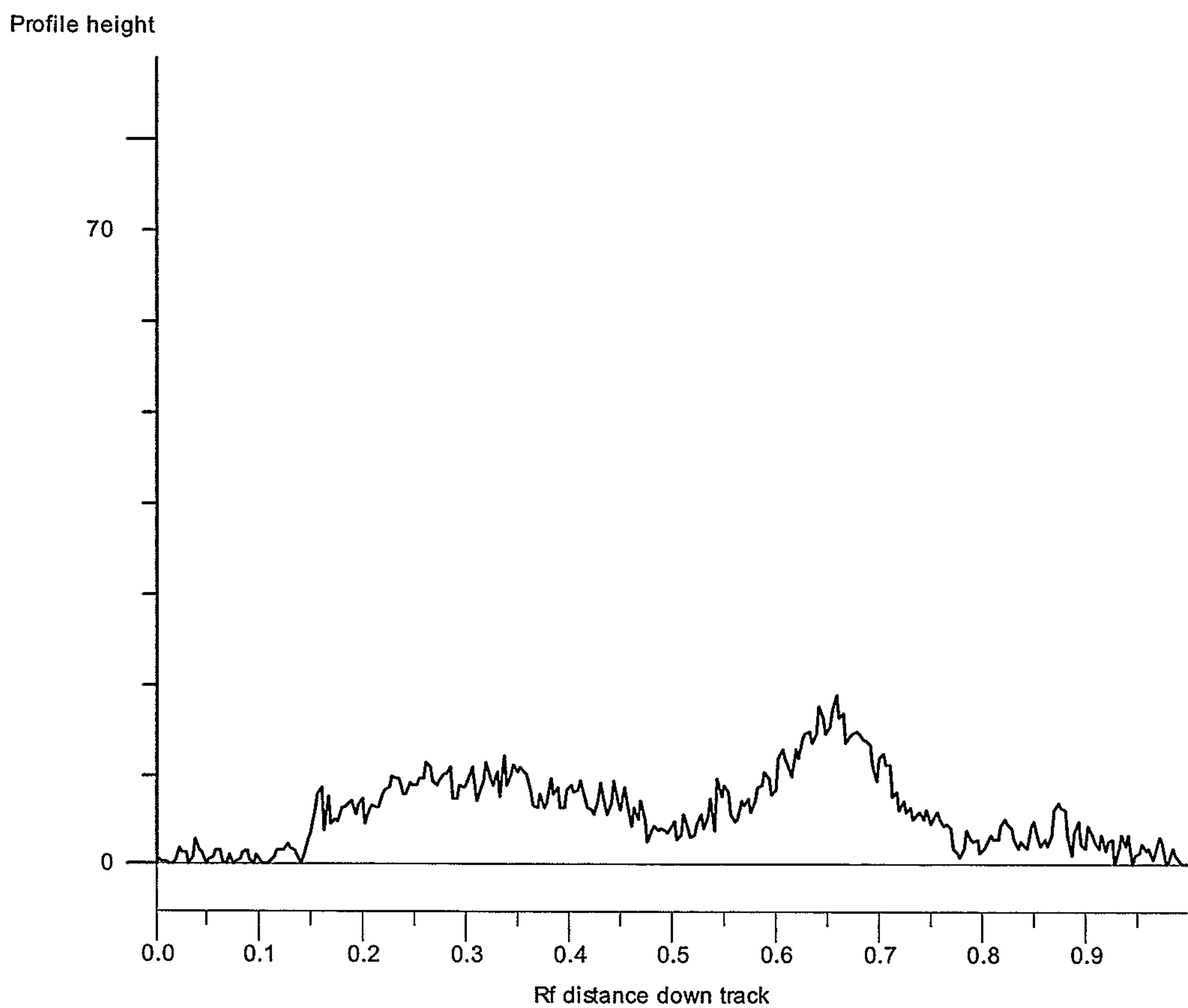
Figure 4. A
GelDoc intensity peaks for expression of GAPDH and OPG in test samples of rat mandible

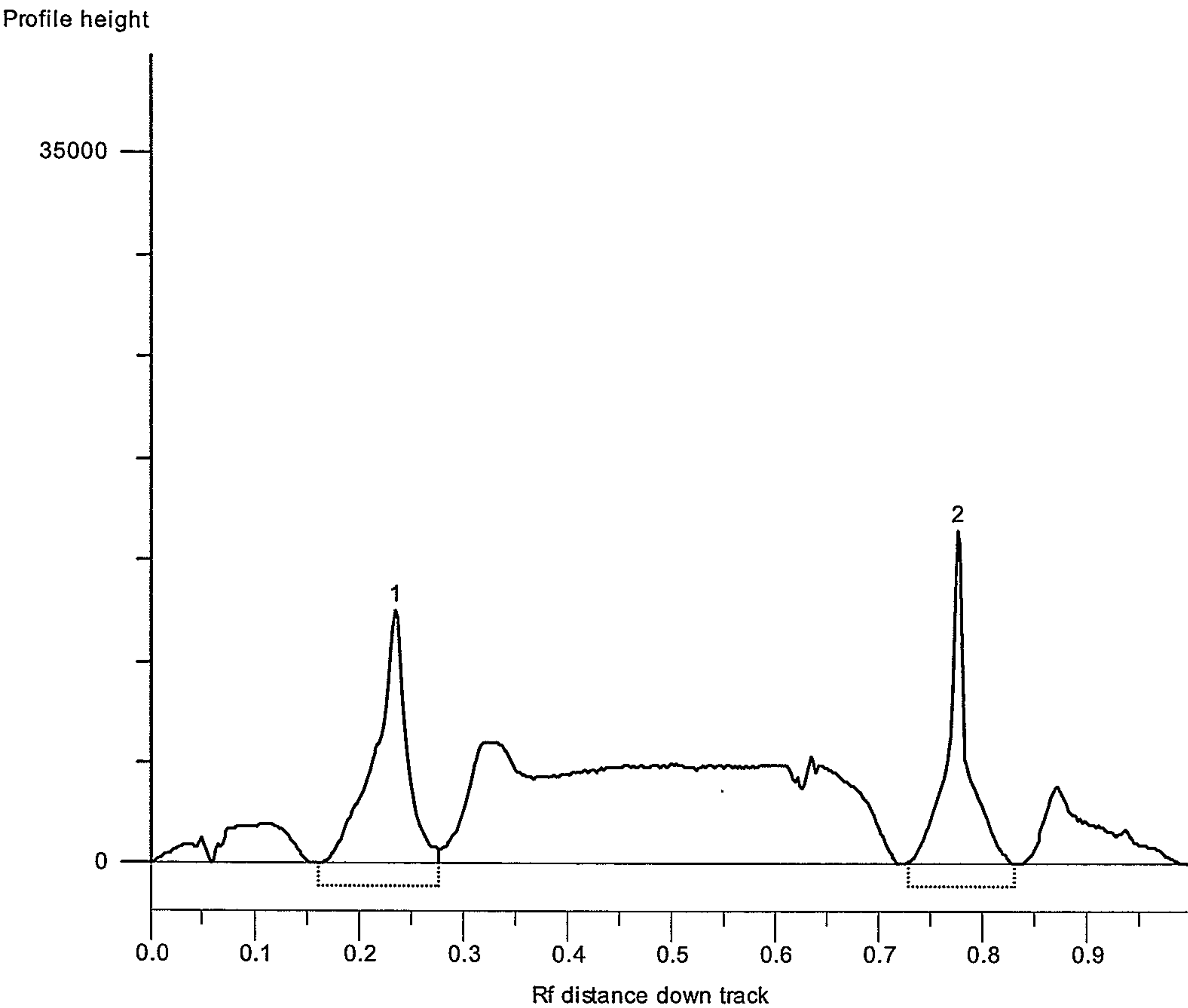
Each graph shows the intensity peak as demonstrated by GeneTools software.

Each table below the graph identifies the Rat from which the sample was taken and indicates the relative quantity of expression of OPG to GAPDH

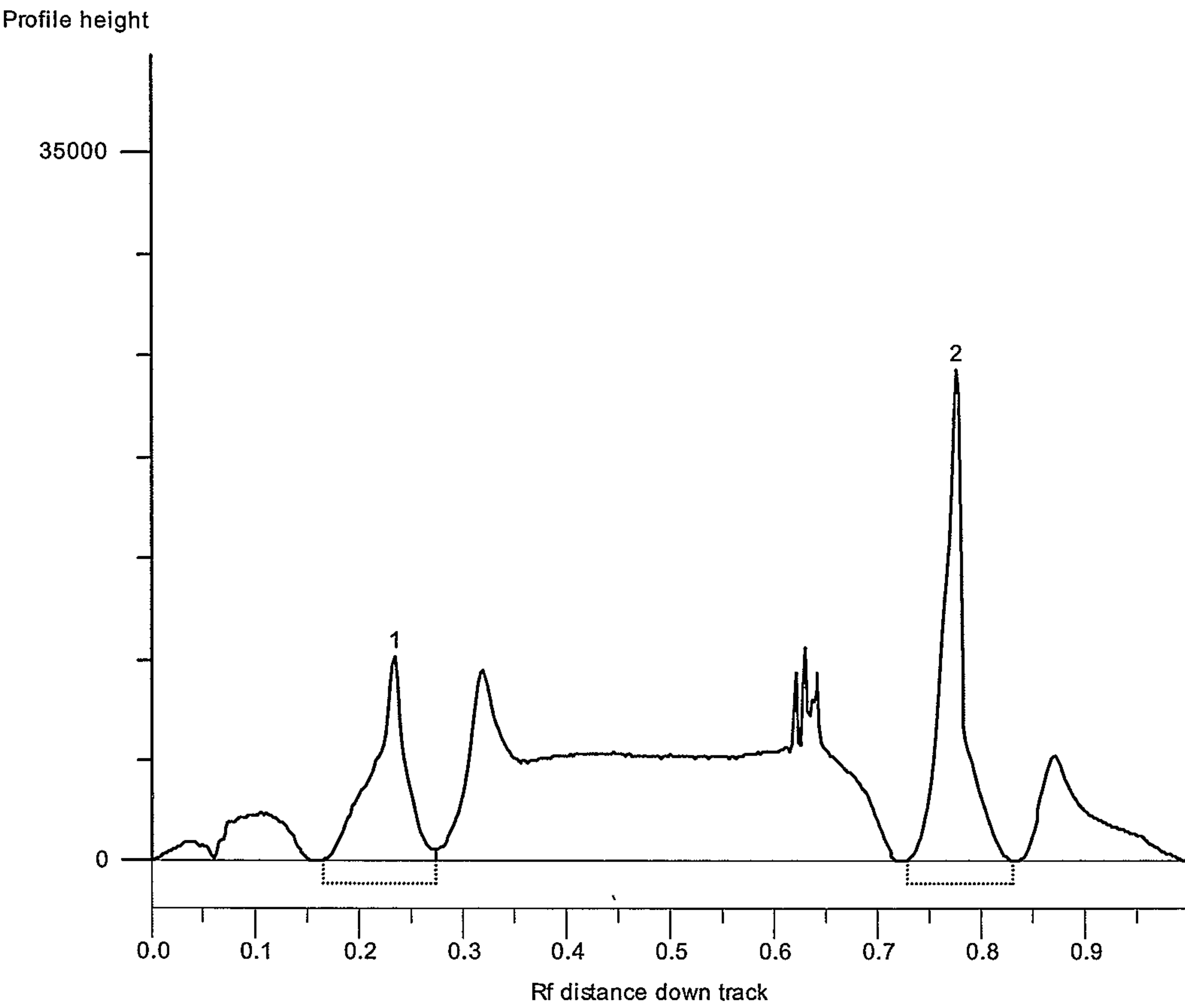
Height indicates the base pair size of product analysed.

Raw volume is the volume assigned by the program as the intensity value of the area of band detected

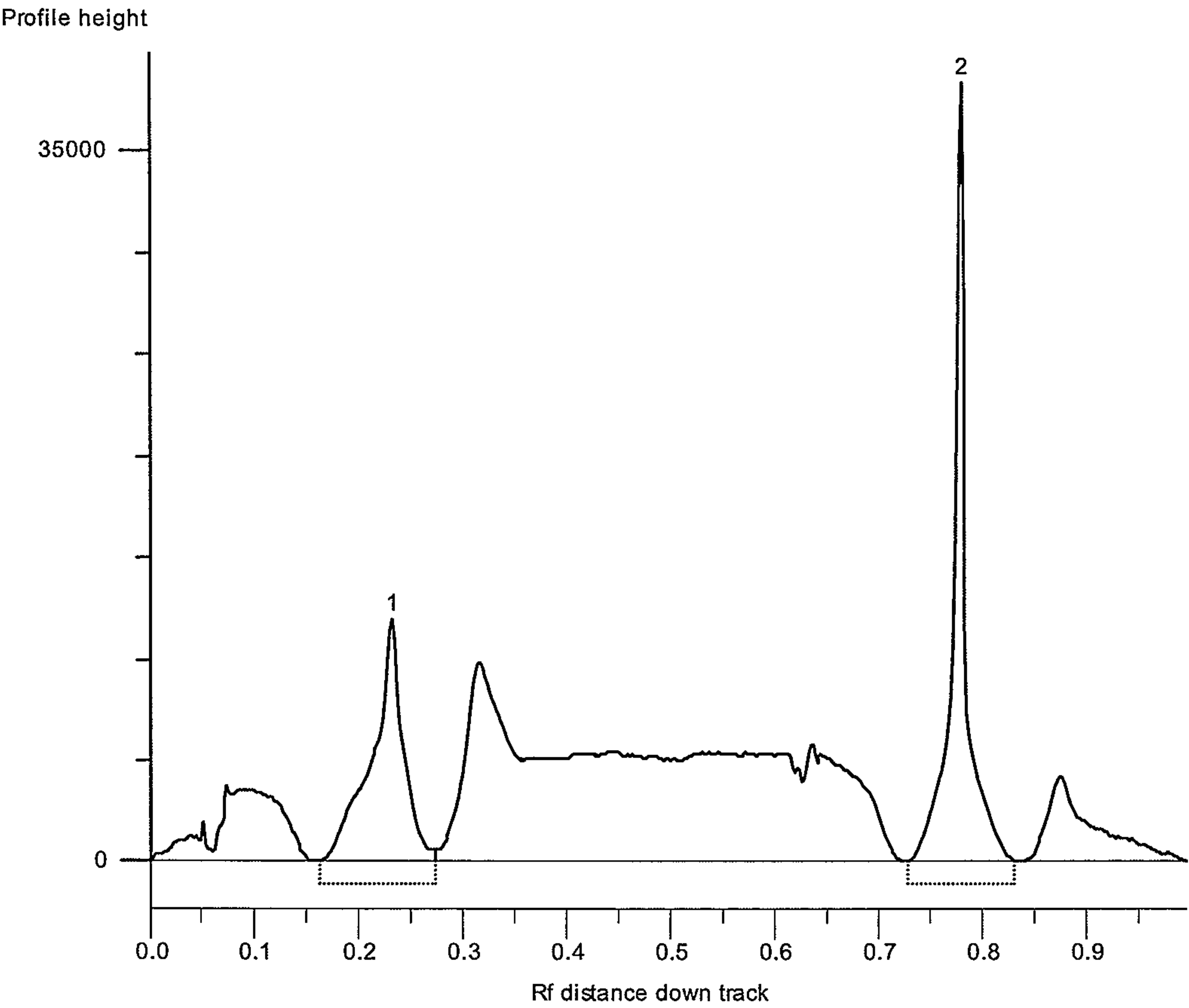




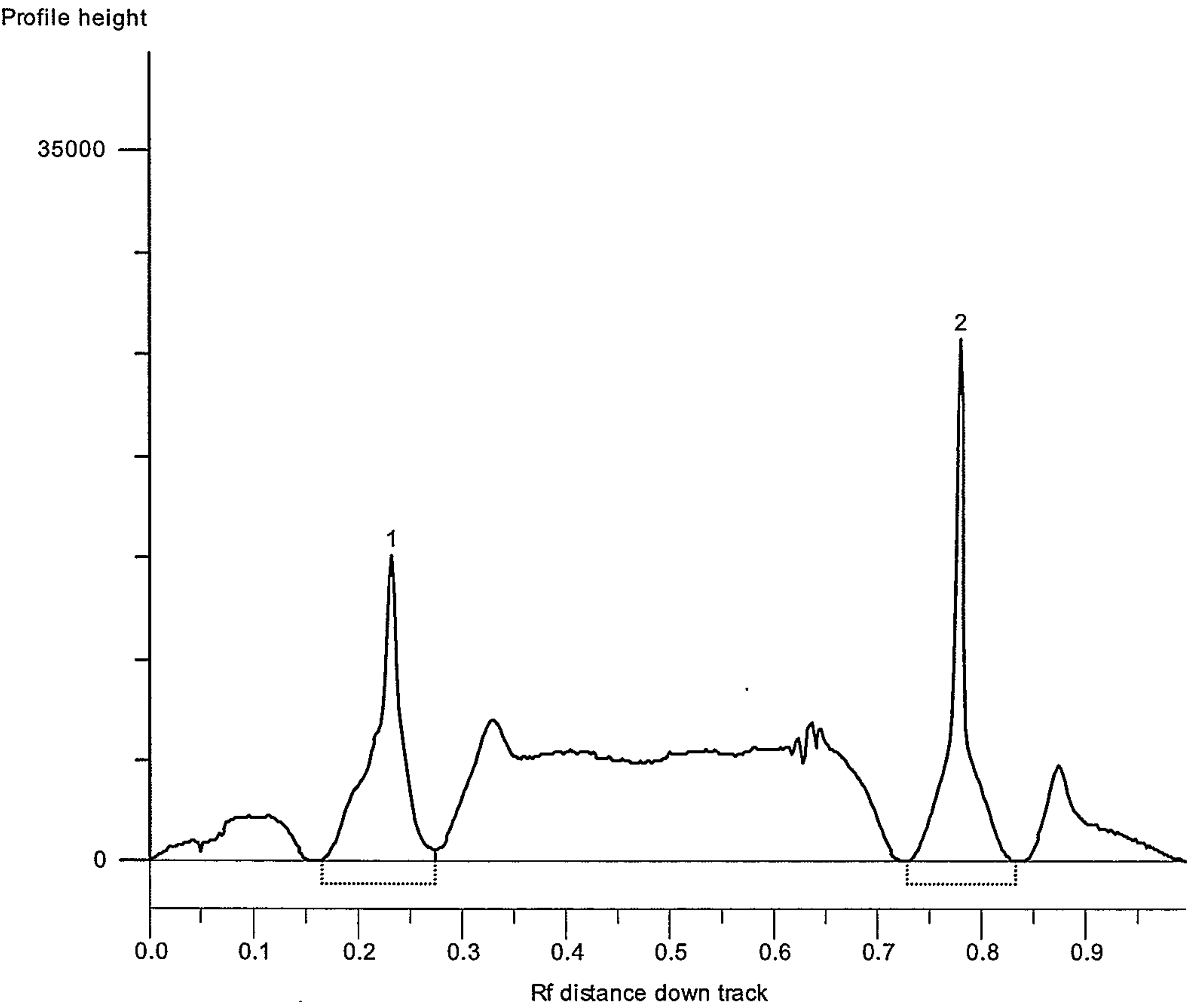
Track 2	Positive control from UMR106 Cells				Ratios of GAPDH/OPG	Ratios of OPG/ GAPDH
Number		Height	Raw vol.	Quantity		
1	GAPDH	12561.63	6420497	2.052949		
2	OPG	16431.63	5200813	1.662956	1.3	0.7



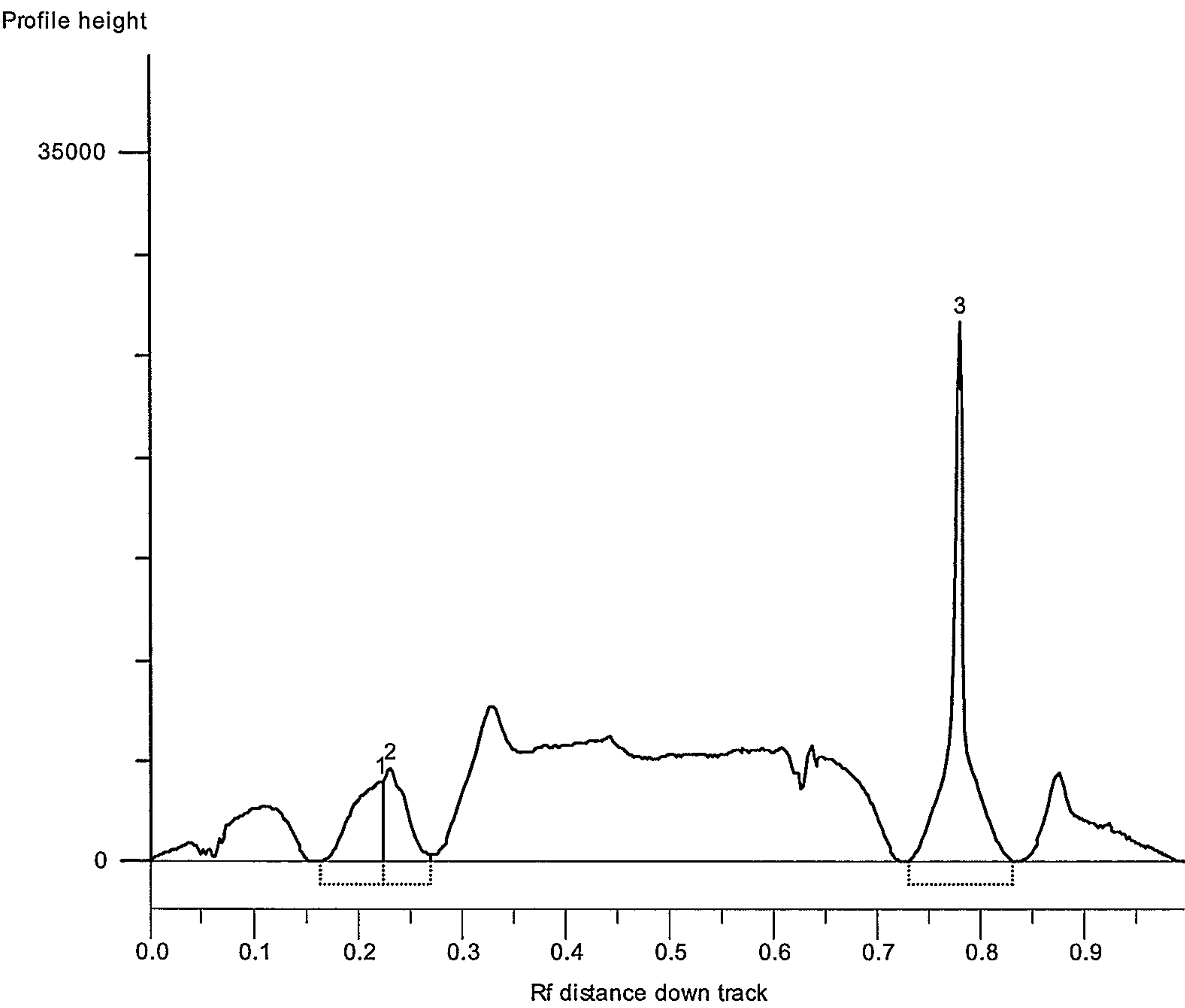
Track 3	Rat 1				Ratios of GAPDH/OPG	Ratios of OPG/ GAPDH
Number		Height	Raw vol.	Quantity		
1	GAPDH	10177.25	5439818	1.739378		
2	OPG	24325.32	8497687	2.71713	0.6	1.67



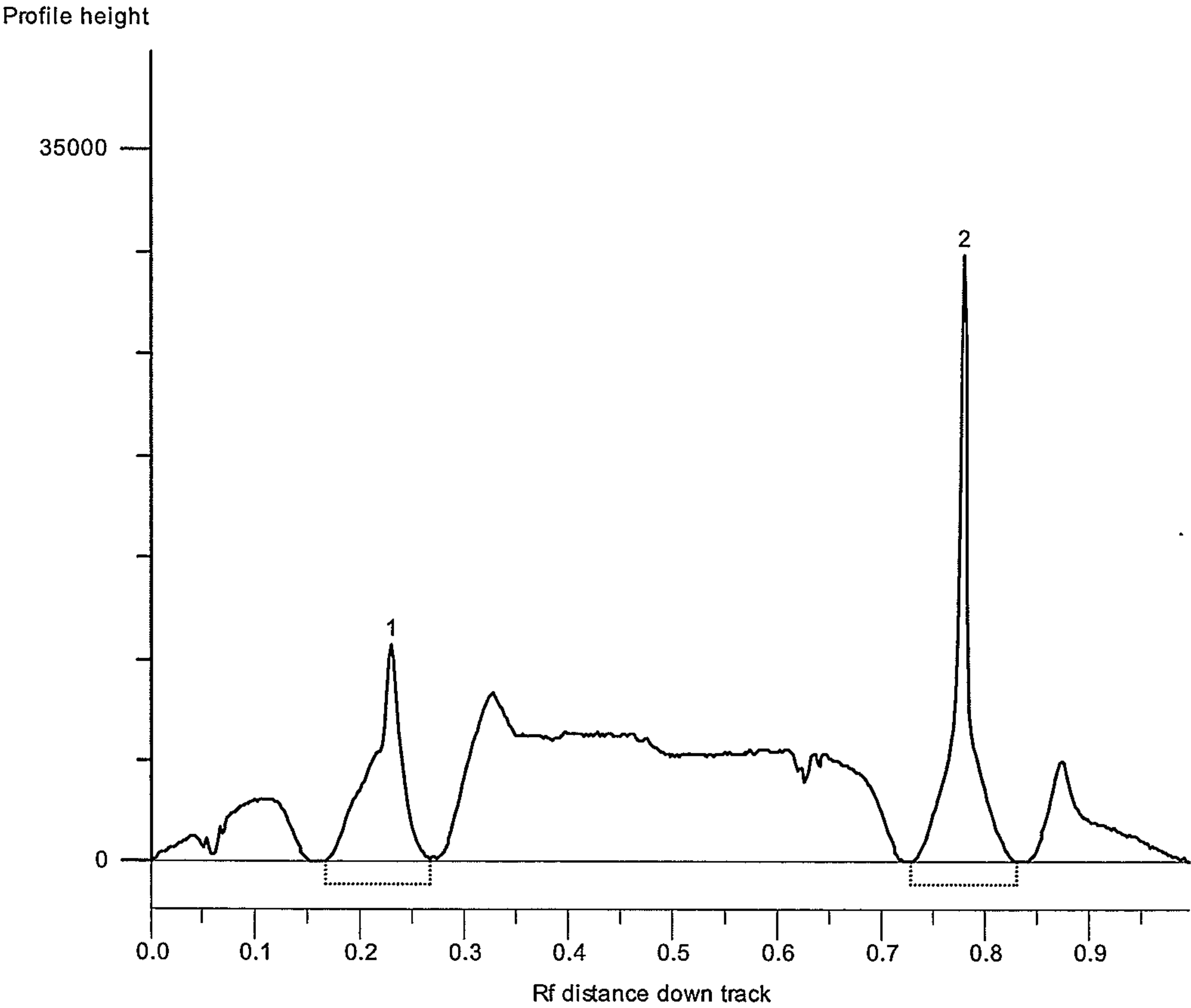
Track 4	Rat 2				Ratios of GAPDH/OPG	Ratios of OPG/ GAPDH
Number		Height	Raw vol.	Quantity		
1	GAPDH	12019.88	6063816	1.938901		
2	OPG	38371.83	8576761	2.742414	0.7	1.4



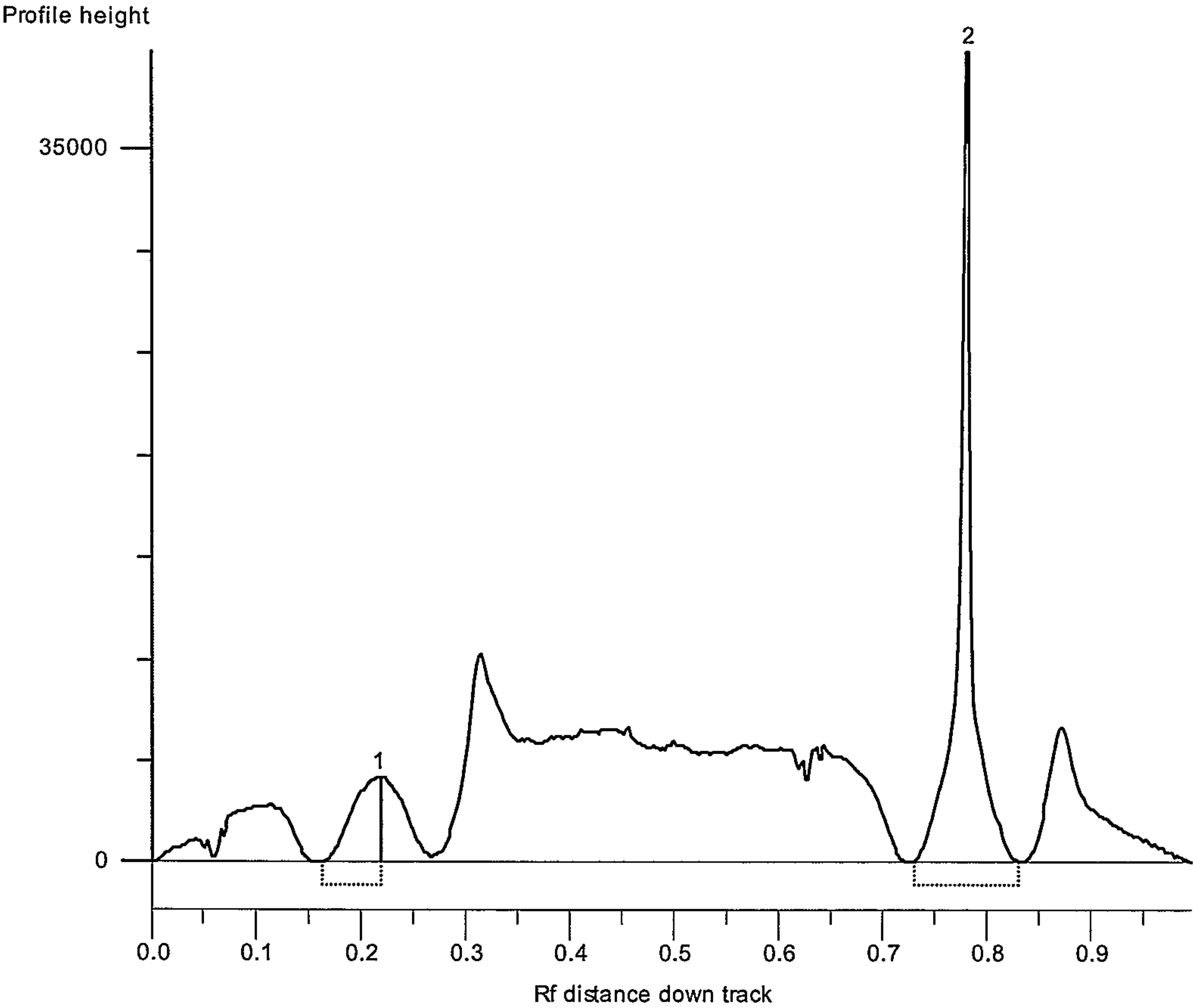
Track 5	Rat 3				Ratios of GAPDH/OPG	Ratios of OPG/ GAPDH
Number		Height	Raw vol.	Quantity		
1	GAPDH	15081.35	6673528	2.133856		
2	OPG	25833.43	6744681	2.156607	1	1



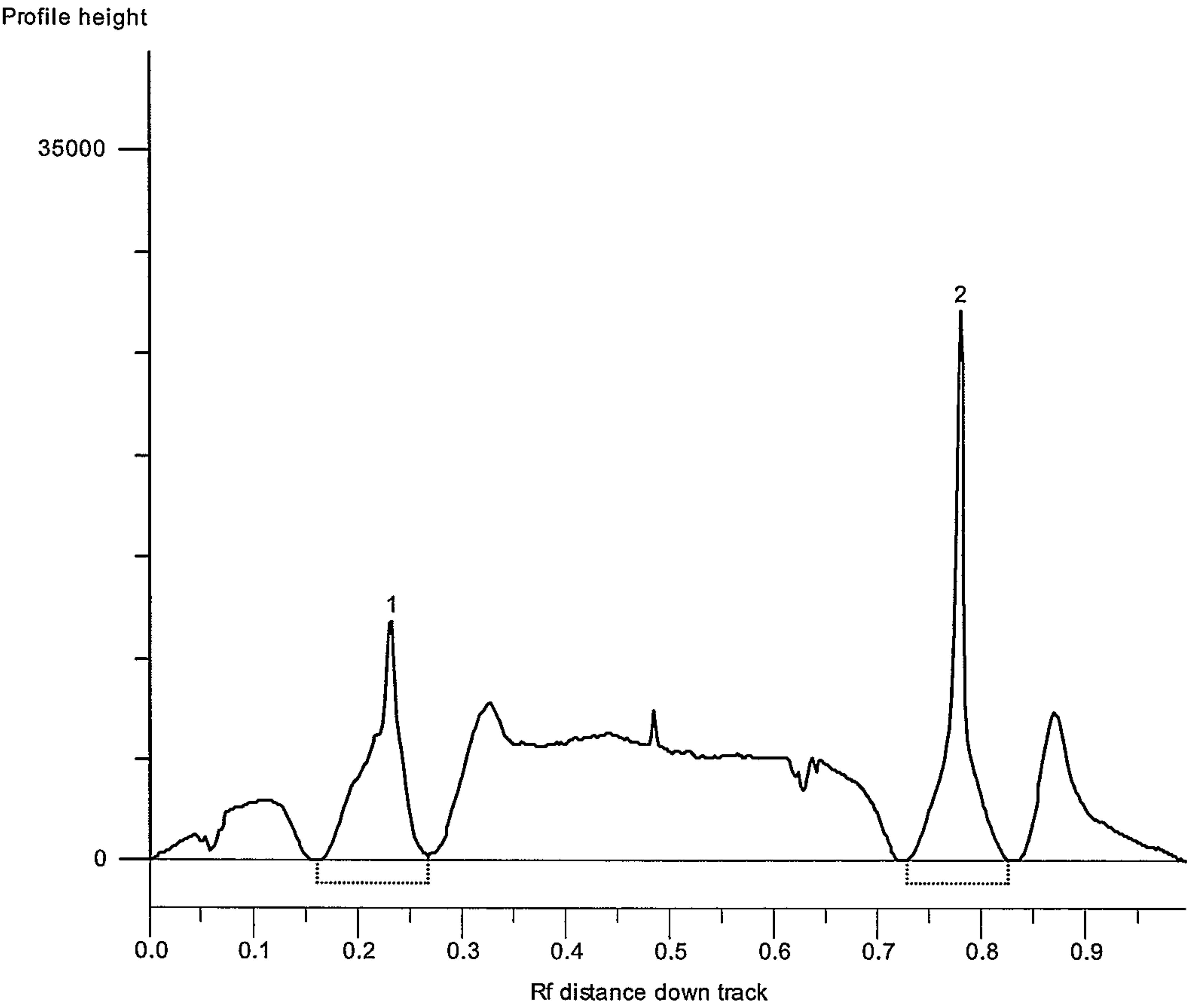
Track 6	Rat 4				Ratios of GAPDH/OPG	Ratios of OPG/ GAPDH
Number		Height	Raw vol.	Quantity		
1	GAPDH	4651.051	1626951	0.520217	0.2	5
2	OPG	26728.48	6645095	2.124764		



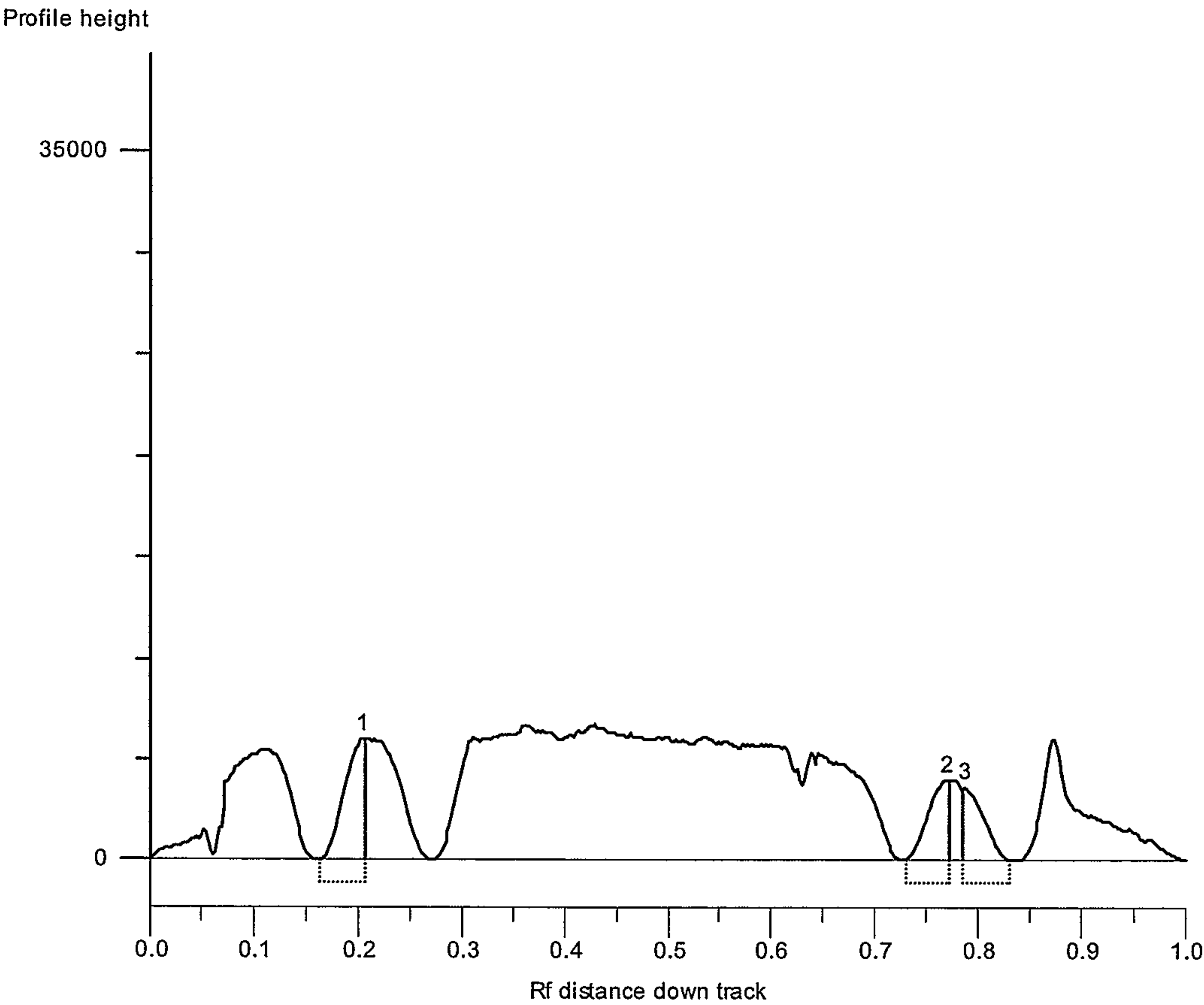
Track 7	Rat 5				Ratios of GAPDH/OPG	Ratios of OPG/ GAPDH
Number		Height	Raw vol.	Quantity		
1	GAPDH	10745.58	5216899	1.6681		
2	OPG	29896.44	7433088	2.376725	0.7	1.4



Track 8	Rat 6				Ratios of GAPDH/OPG	Ratios of OPG/ GAPDH
Number		Height	Raw vol.	Quantity		
1	GAPDH	4263.139	2011672	0.643231		
2	OPG	39916.82	9699384	3.101371	0.2	5



Track 9	Rat 7				Ratios of GAPDH/OPG	Ratios of OPG/ GAPDH
Number		Height	Raw vol.	Quantity		
1	0	11849.88	5922525	1.893723		
2	0	27194.49	7109007	2.2731	0.8	1.25



Track 10	Negative control no expression seen
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Figure 4B

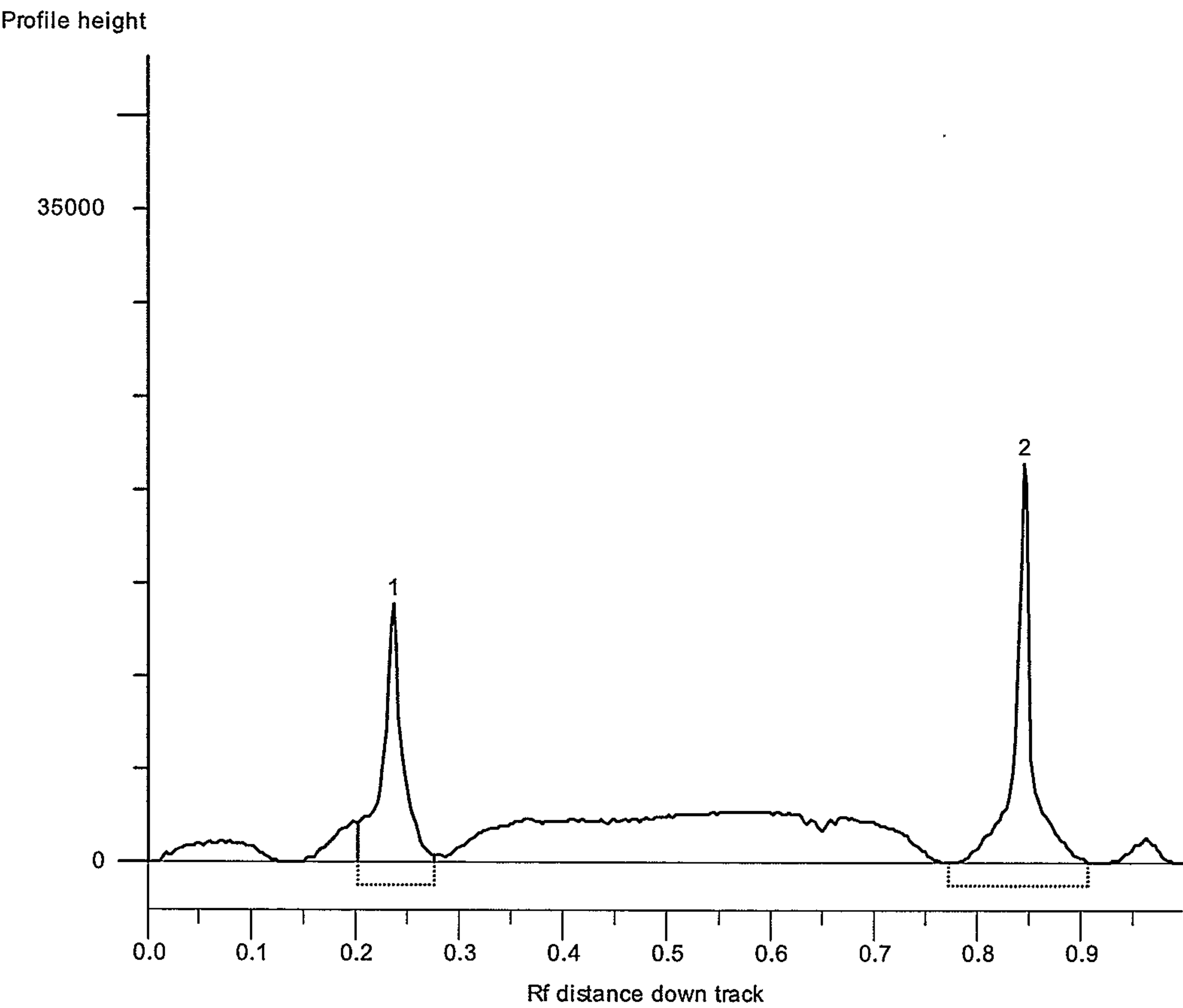
GelDoc intensity peaks for expression of GAPDH and OPG in control samples of rat mandible

Each graph shows the intensity peak as demonstrated by GeneTools software.

Each table below the graph identifies the Rat from which the sample was taken and indicates the relative quantity of expression of OPG to GAPDH

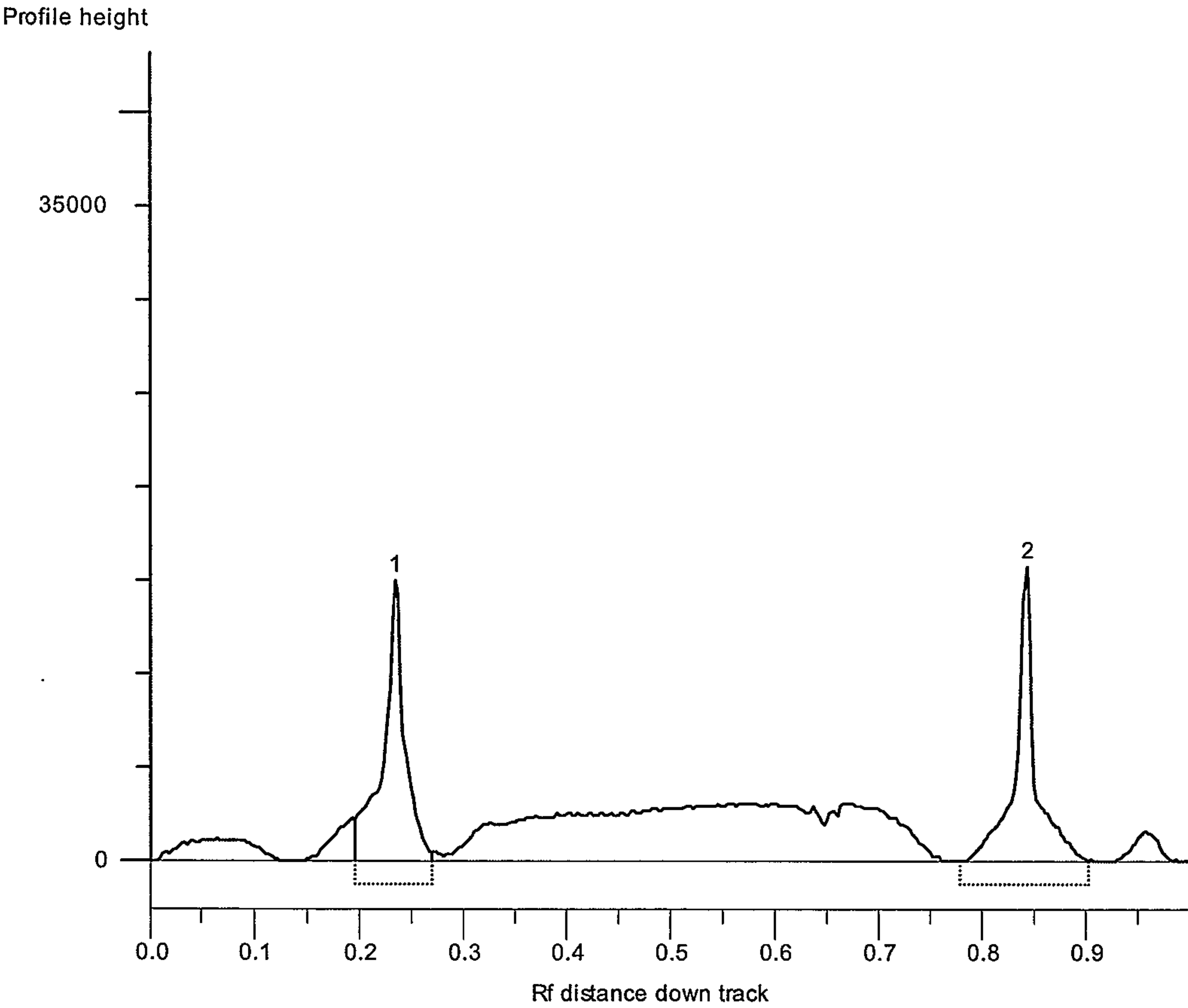
Height indicates the base pair size of product analysed.

Raw volume is the volume assigned by the program as the intensity value

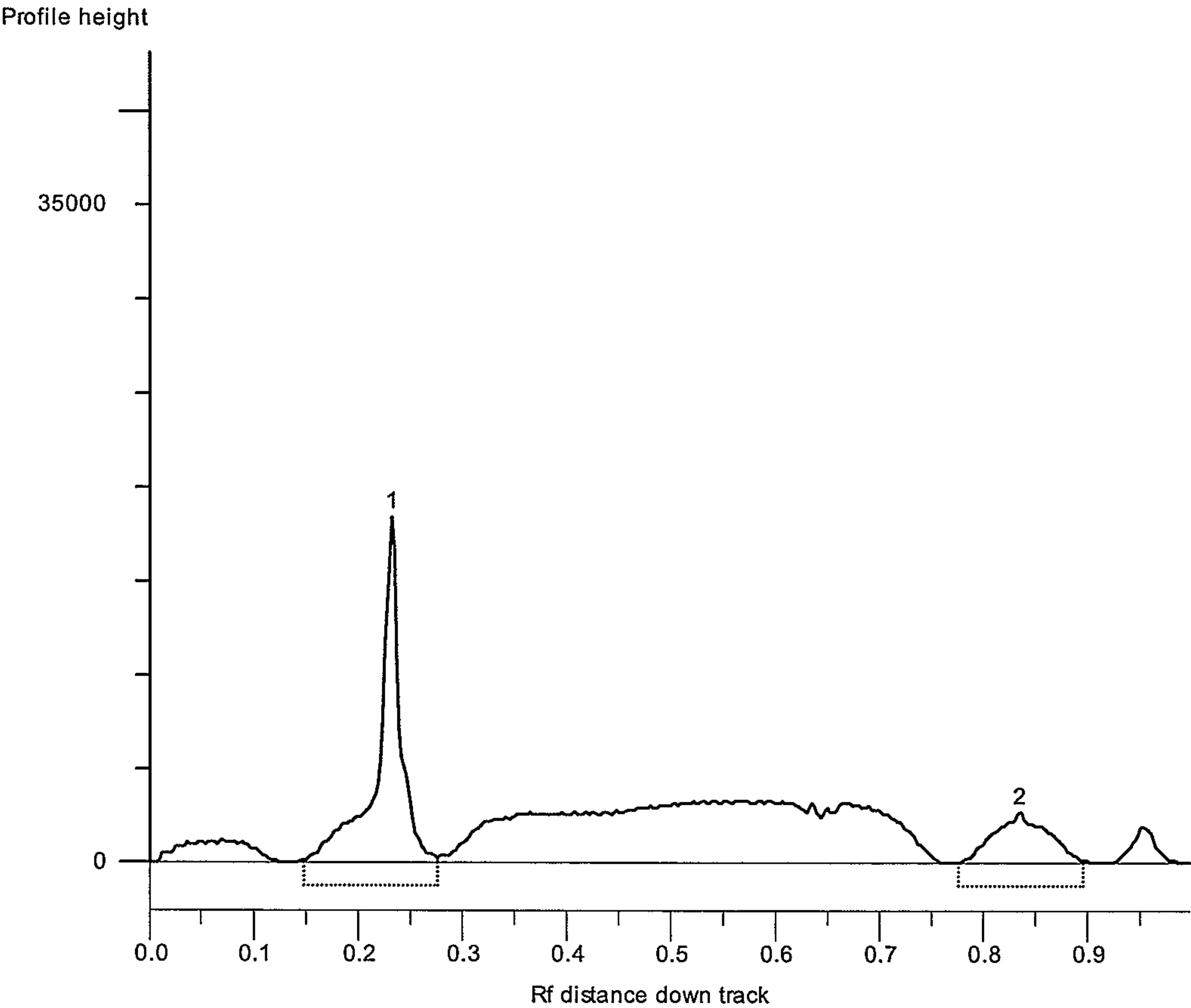


Track 2	Positive control				Ratios of	Ratios of OPG/
	UMR106 cells				GAPDH/OPG	GAPDH
Number		Height	Raw vol.	Quantity		

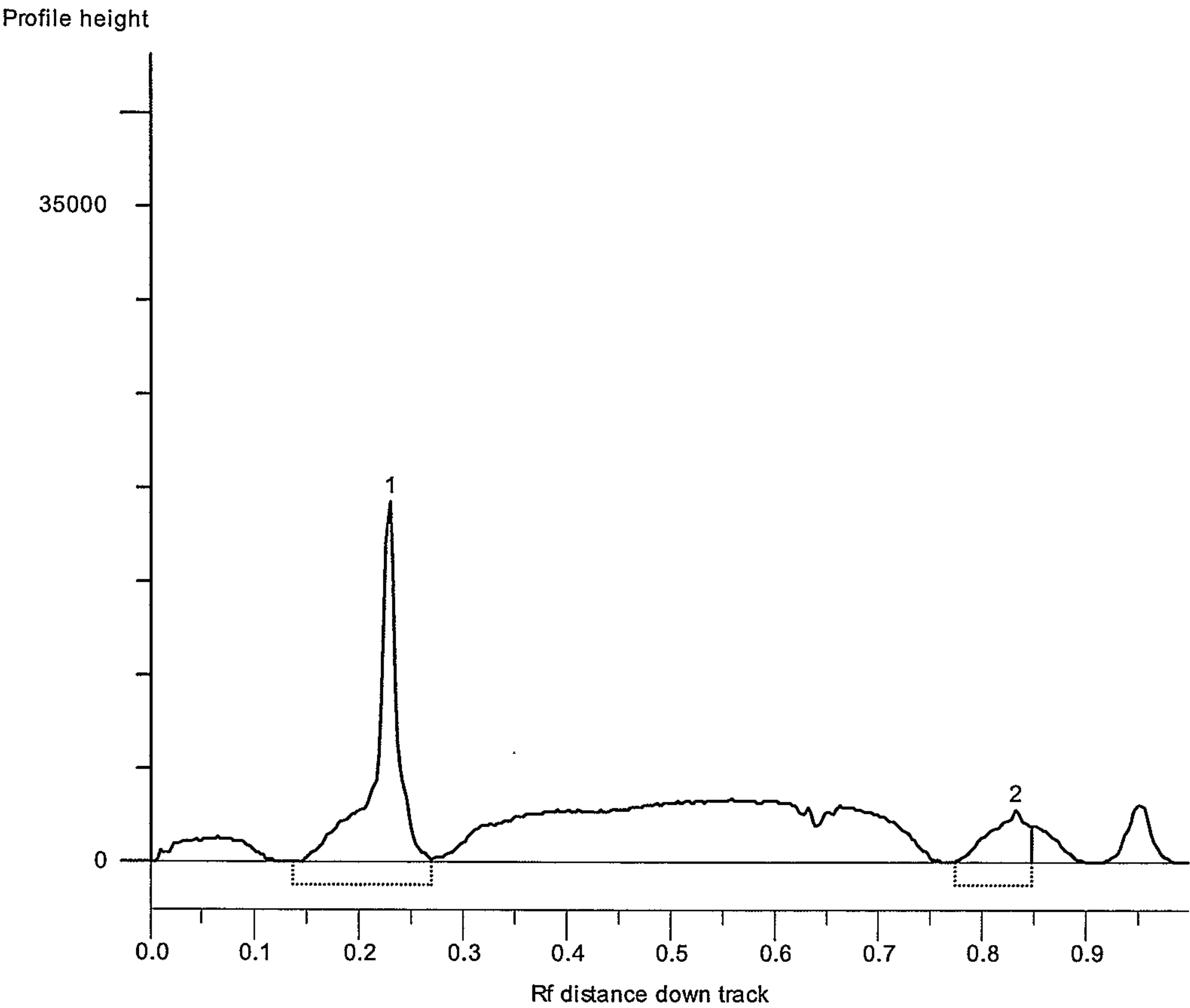
1	GAPDH	13881.69	3830071	2.3269		
2	OPG	21432.52	5034459	3.058607	0.75	1.3



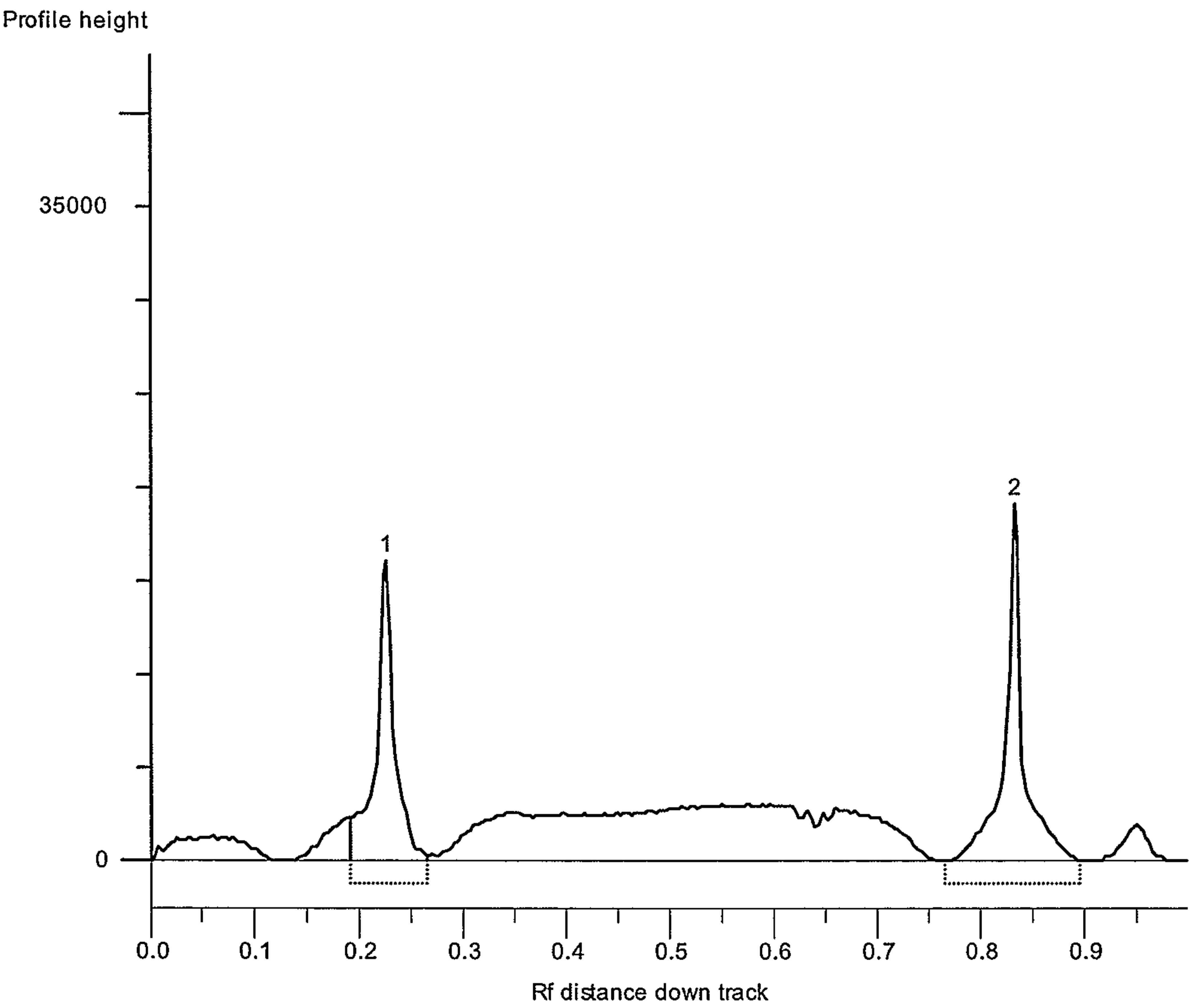
Track 3					Ratios of GAPDH/OPG	Ratios of OPG/ GAPDH
Number		Height	Raw vol.	Quantity		
1	GAPDH	15014.09	4367557	2.653442		
2	OPG	15736.55	4151743	2.522327	1.04	0.9



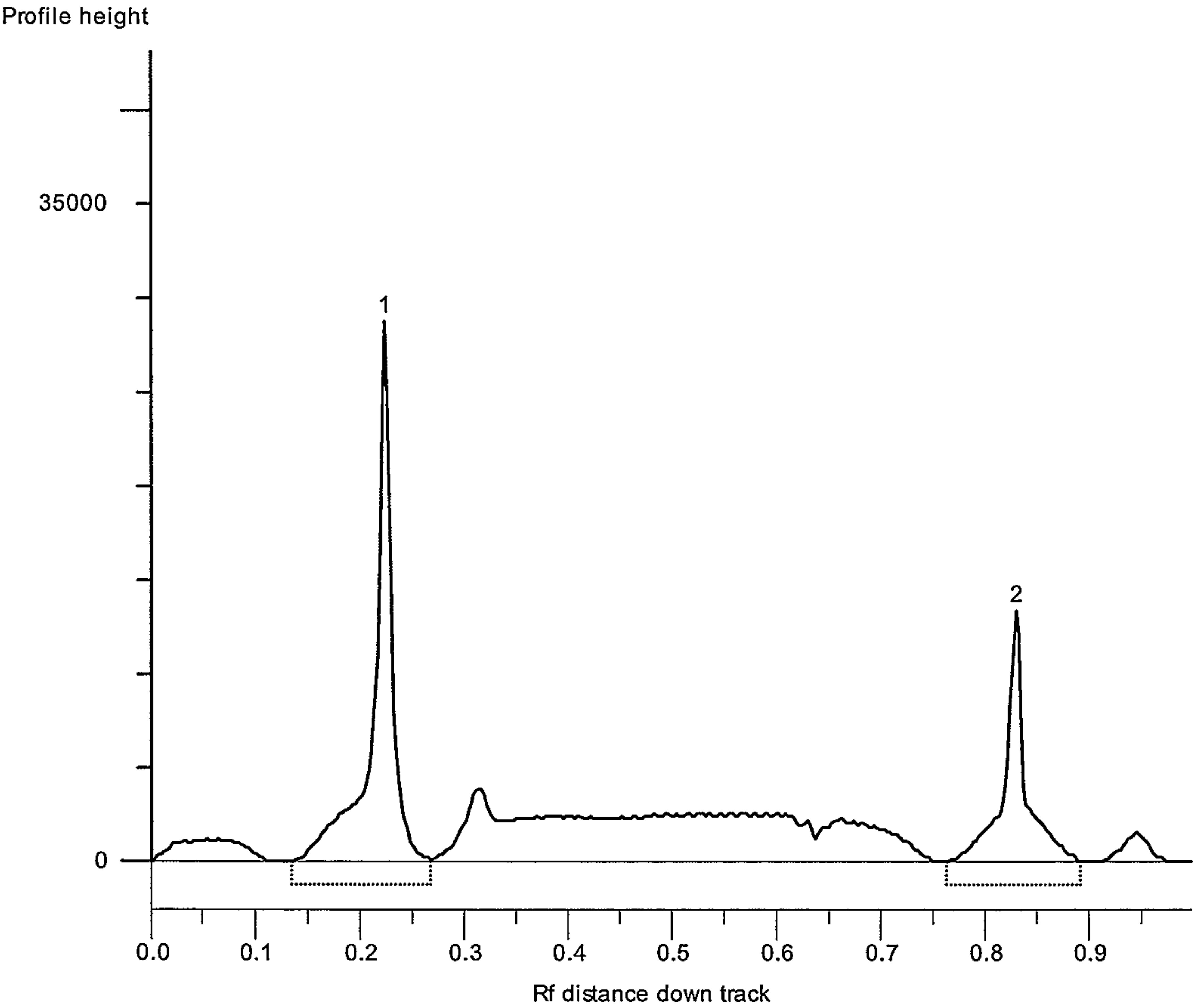
Track 4					Ratios of GAPDH/OPG	Ratios of OPG/ GAPDH
Number		Height	Raw vol.	Quantity		
1	GAPDH	18426.23	5400531	3.281009		
2	OPG	2769.802	2021965	1.228413	2.6	0.38



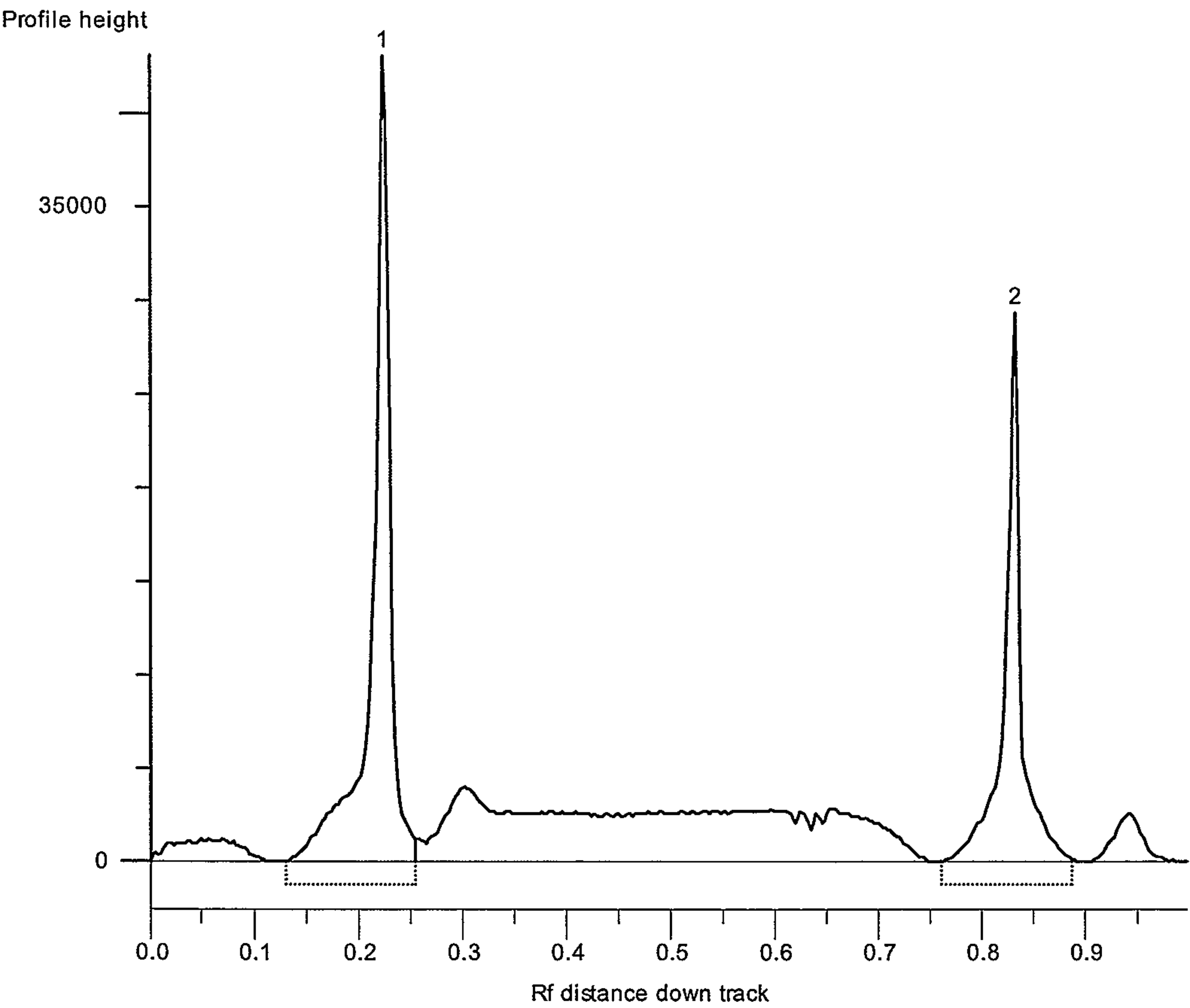
Track 5					Ratios of GAPDH/OPG	Ratios of OPG/ GAPDH
Number		Height	Raw vol.	Quantity		
1	GAPDH	19290.63	5425142	3.295961		
2	OPG	2833.65	1426352	0.866558	4	0.25



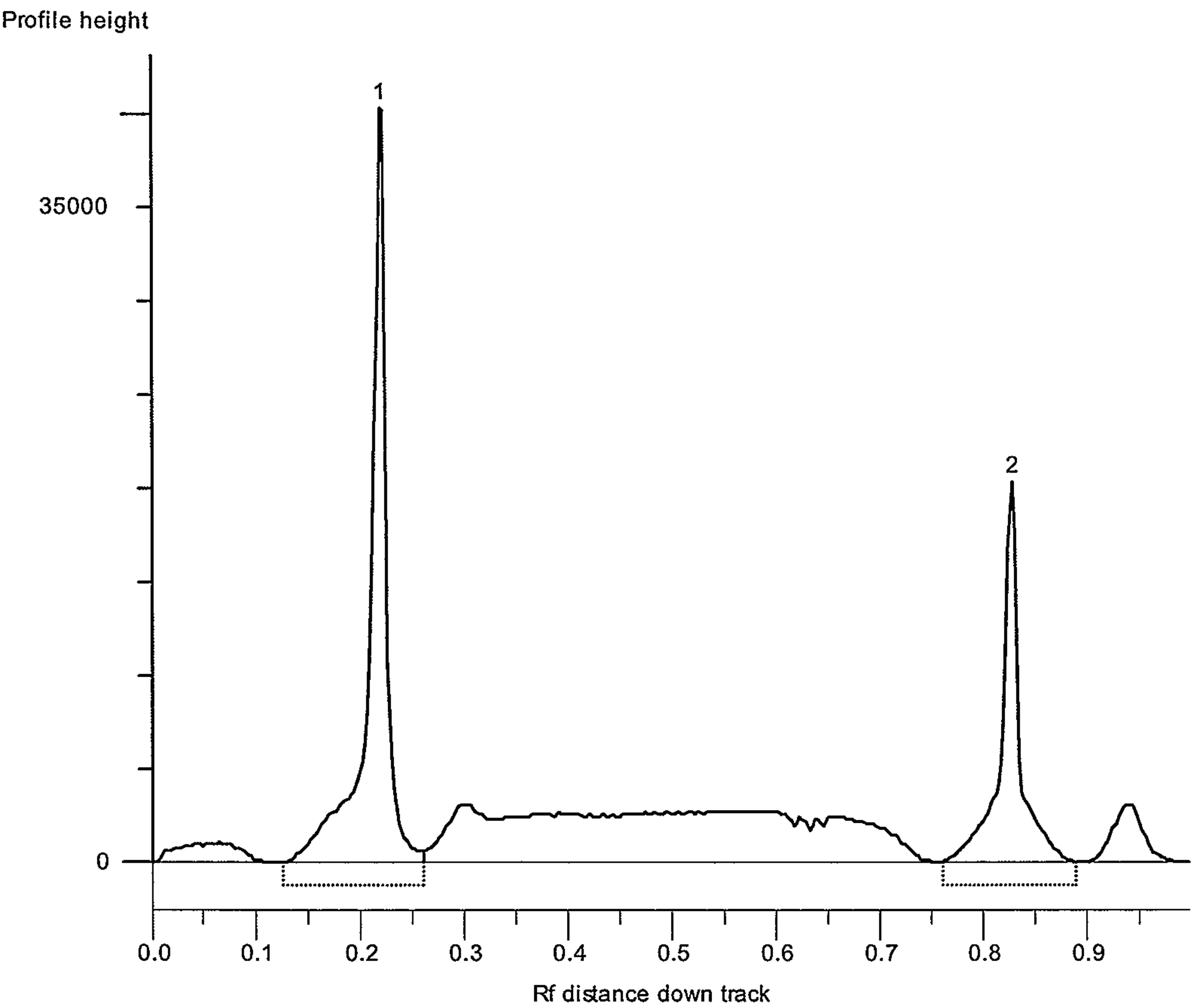
Track 6					Ratios of GAPDH/OPG	Ratios of OPG/ GAPDH
Number		Height	Raw vol.	Quantity		
1	GAPDH	16128.36	4034447	2.451066		
2	OPG	19129.09	4802158	2.917477	0.8	1.25



Track 7					Ratios of GAPDH/OPG	Ratios of OPG/ GAPDH
Number		Height	Raw vol.	Quantity		
1	GAPDH	28776.3	6933659	2.212437		
2	OPG	13509.86	3867094	2.349393	1	1



Track 8					Ratios of GAPDH/OPG	Ratios of OPG/ GAPDH
Number		Height	Raw vol.	Quantity		
1	GAPDH	43227.07	10180983	6.185298		
2	OPG	29425.54	6779392	4.118715	1.4	0.7



Track 9					Ratios of GAPDH/OPG	Ratios of OPG/ GAPDH
Number		Height	Raw vol.	Quantity		
1	GAPDH	40310.55	9243723	5.615881		
2	OPG	20381.9	5065990	3.077764	1.5	0.6

Figure 5

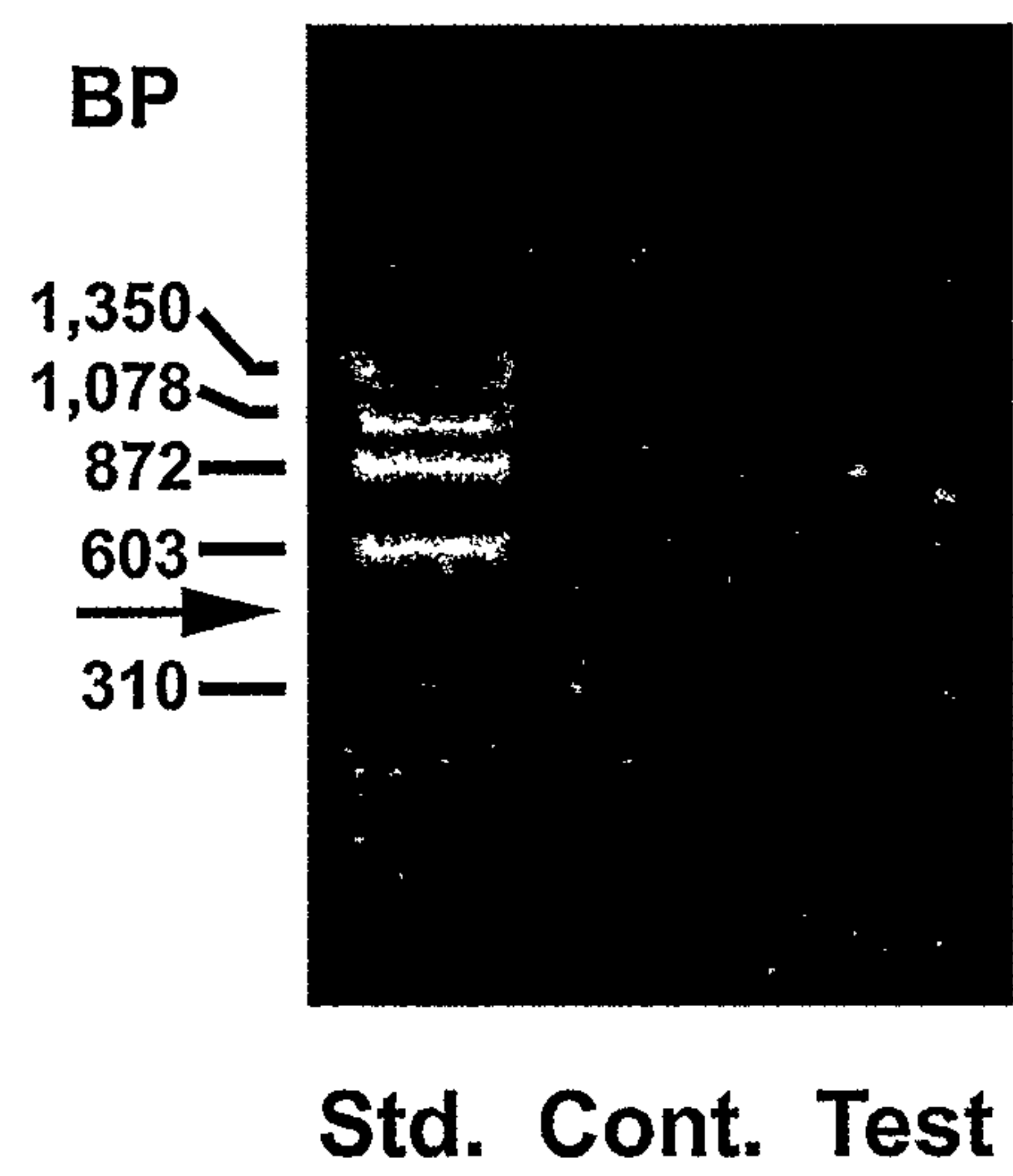


Figure 6A and B

RANKL amplification form two Rat mandibles

Each figure shows 3 lanes

Lane 1: molecular weight marker, lane 2 is amplification signal from RNA collected form control side of rat mandible, lane 3 is amplification signal from RNA collected form test side of rat mandible

Figure 6A:

Rat 1

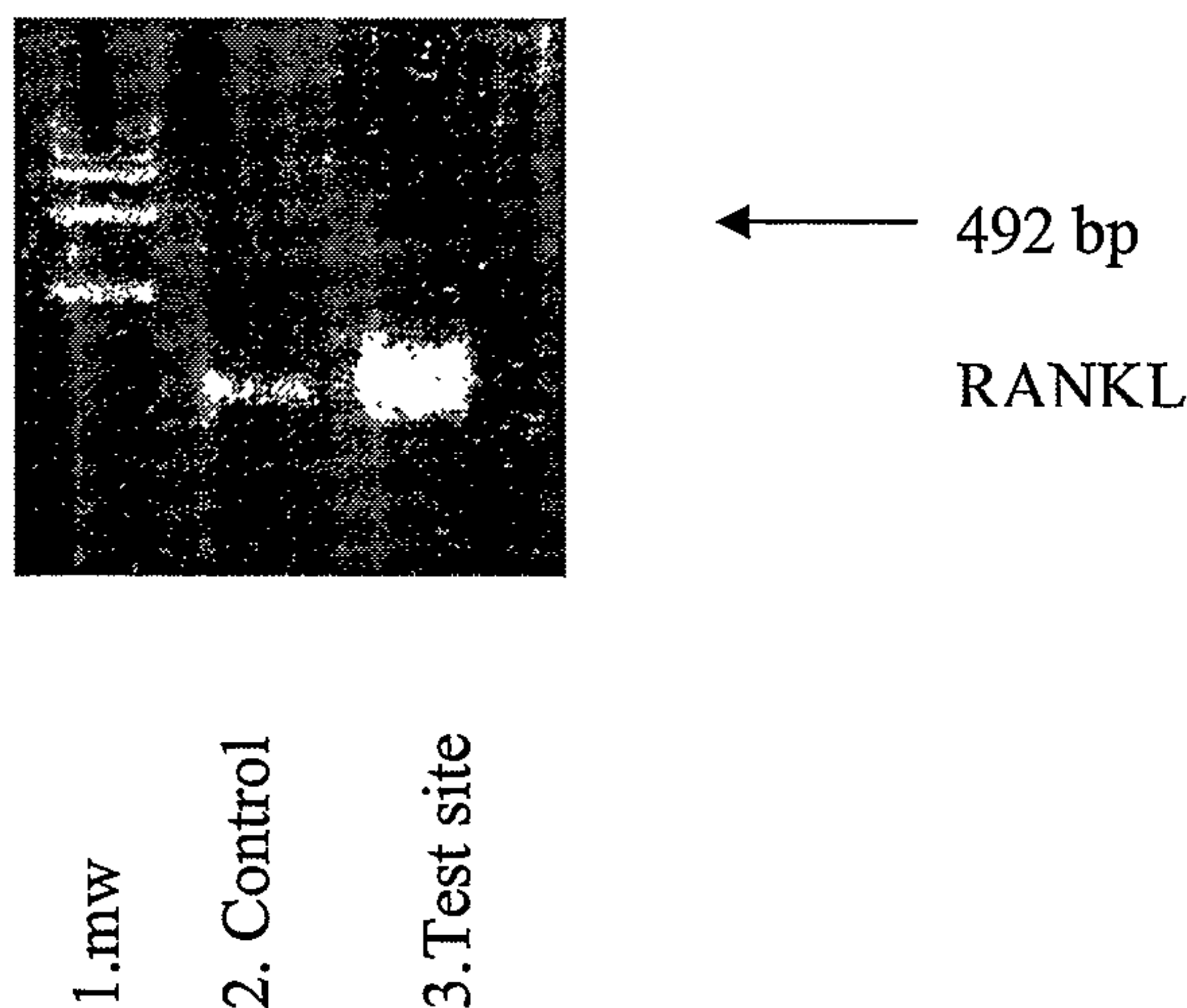


Figure shows agarose gel electrophoresis of RT-PCR of RANKL from test and control sites of RAT Number 2

Lane 1 is molecular weight marker IX

Lane 2 – amplification of mRNA from control site no amplification is seen

Lane 3 – amplification f mRNA from test site showing amplification product of 492 bp

Figure 6B:

Rat 4

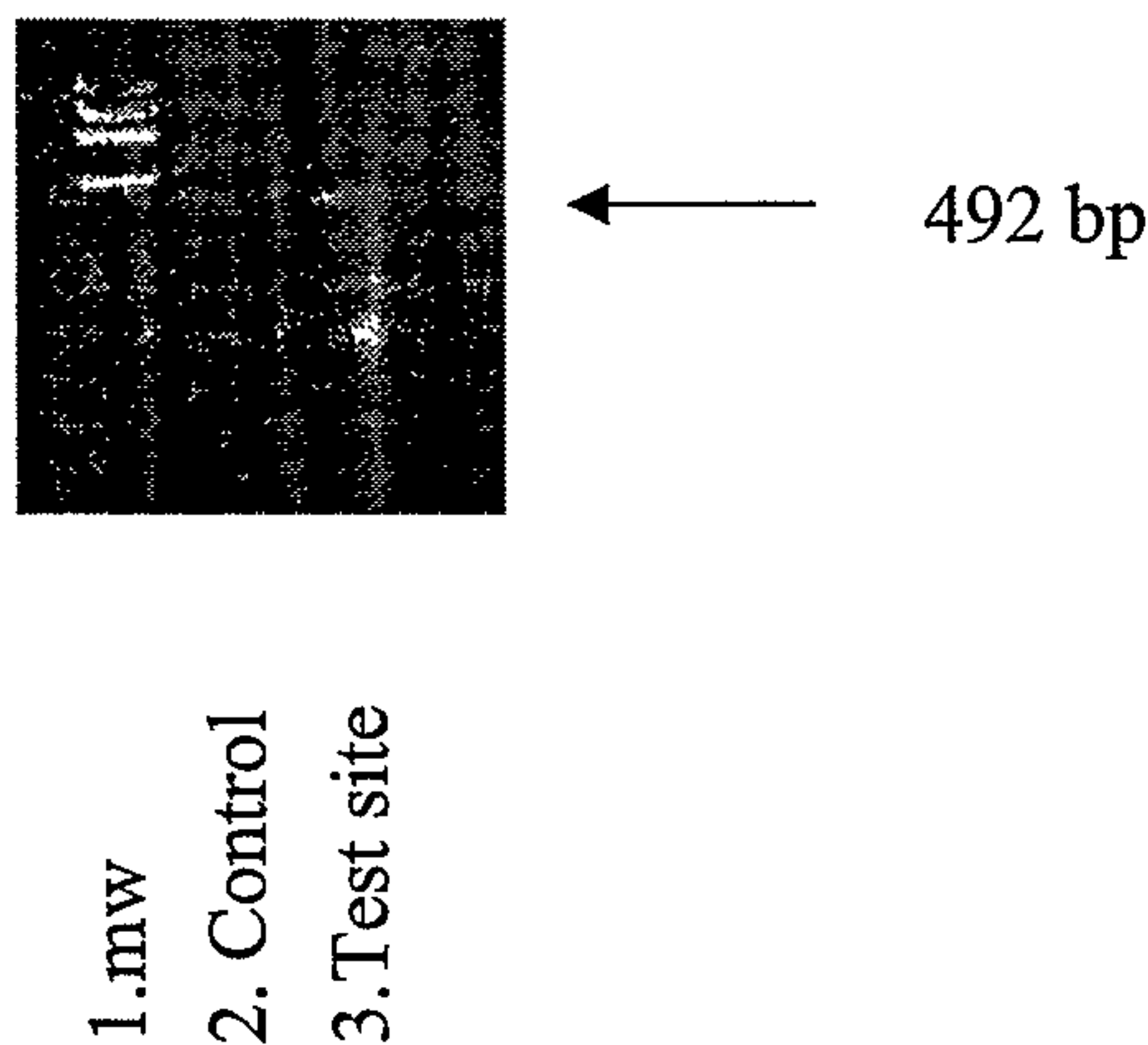


Figure shows agarose gel electrophoresis of RT-PCR of RANKL from test and control sites of RAT number 3

Lane 1 is molecular weight marker IX

Lane 2 – amplification of mRNA from control site no amplification is seen

Lane 3 – amplification f mRNA from test site showing amplification product of 492 bp

Figure 7

RANKL expression levels as analysed by Gene Tools

Graphs generated from Gene Tools program. Each graph shows intensity signals from 4 different rats in relation to a standard.

Each graph shows the intensity peak as demonstrated by GeneTools software.

Each table below the graph identifies the Rat from which the sample was taken and indicates the relative quantity of expression of RANK comparing test and control side of each animal

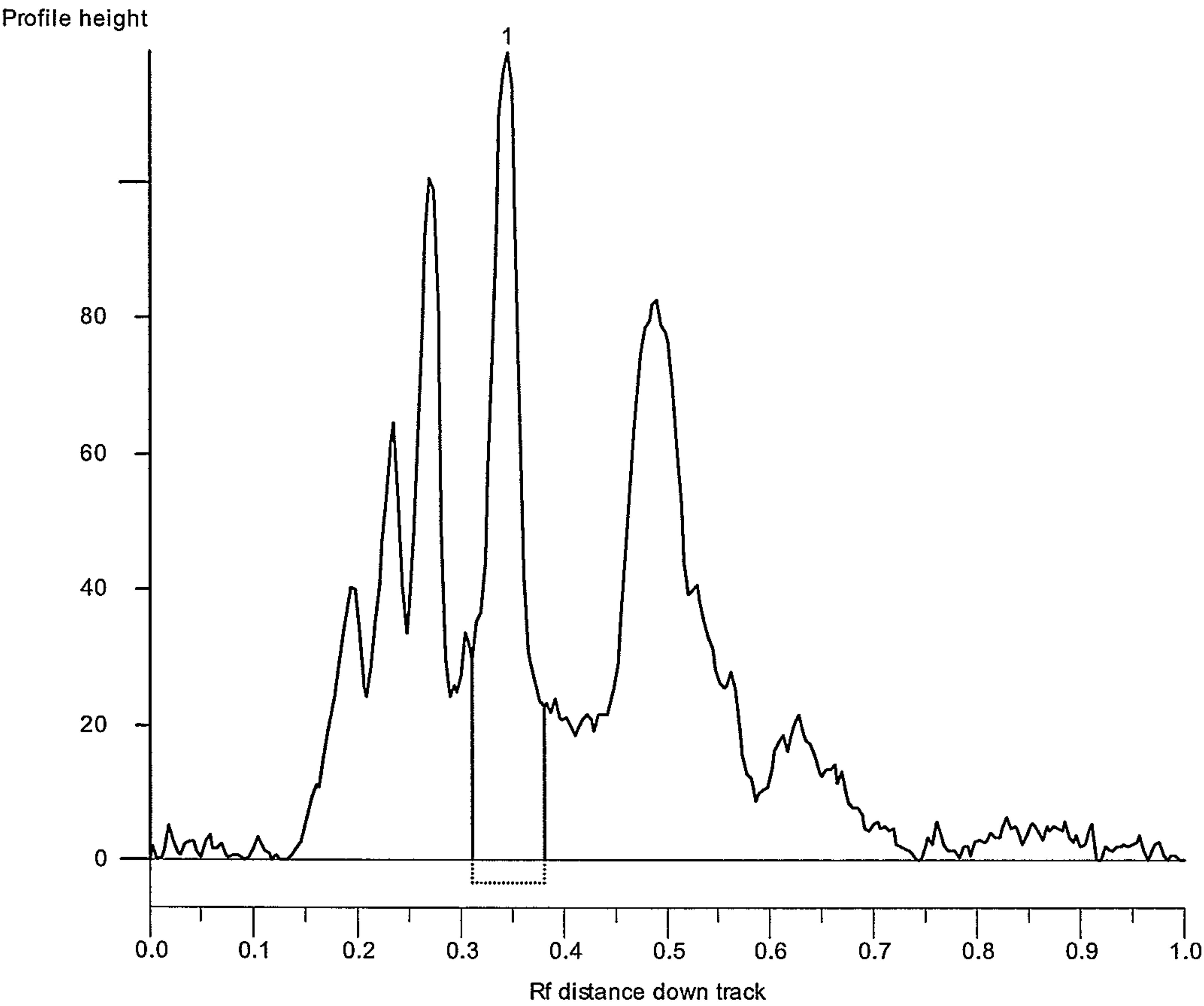
Height indicates the base pair size of product analysed.

Raw volume is the volume assigned by the program as the intensity value of the area of band detected

Graphs 1-3 show results from Rat 1

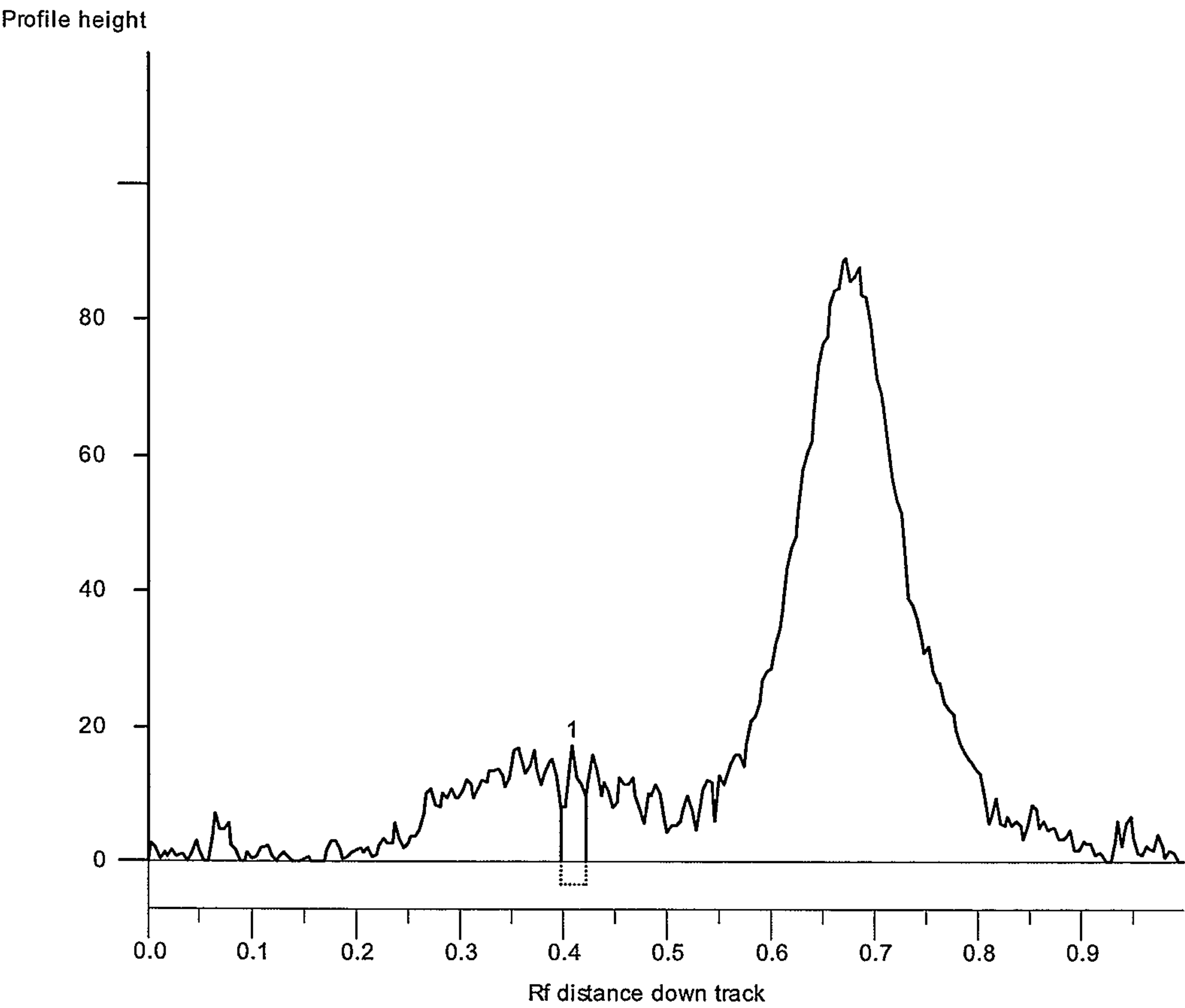
Graphs 4-8 show results from Rat 2 and 3

Graph 9-11 show results from Rat 4



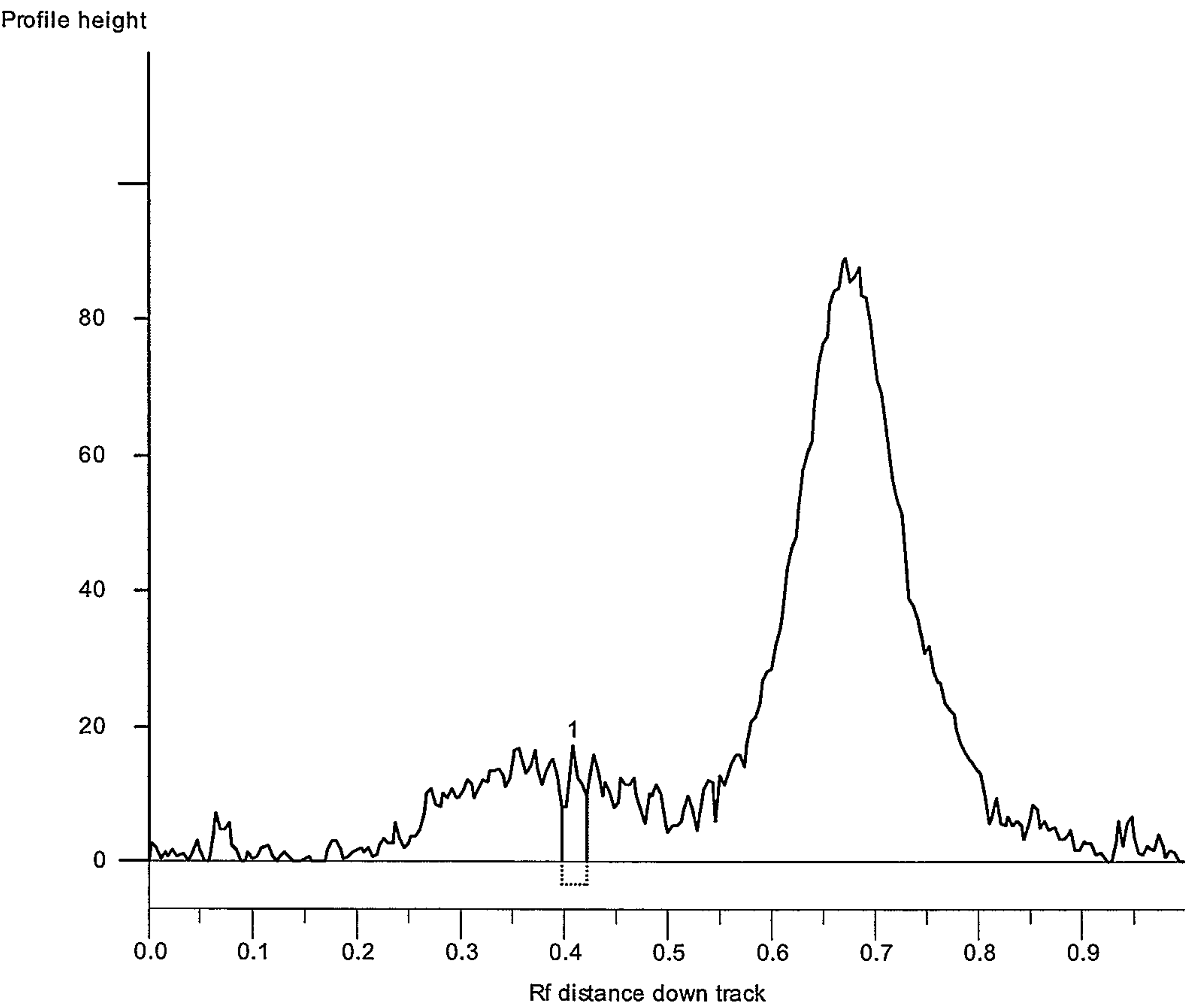
Graph 1

Track 1	Standard peak			
Number	Mol. weight	Height	Raw vol.	Quantity
1	0.00	119.254	66518.07	1.00



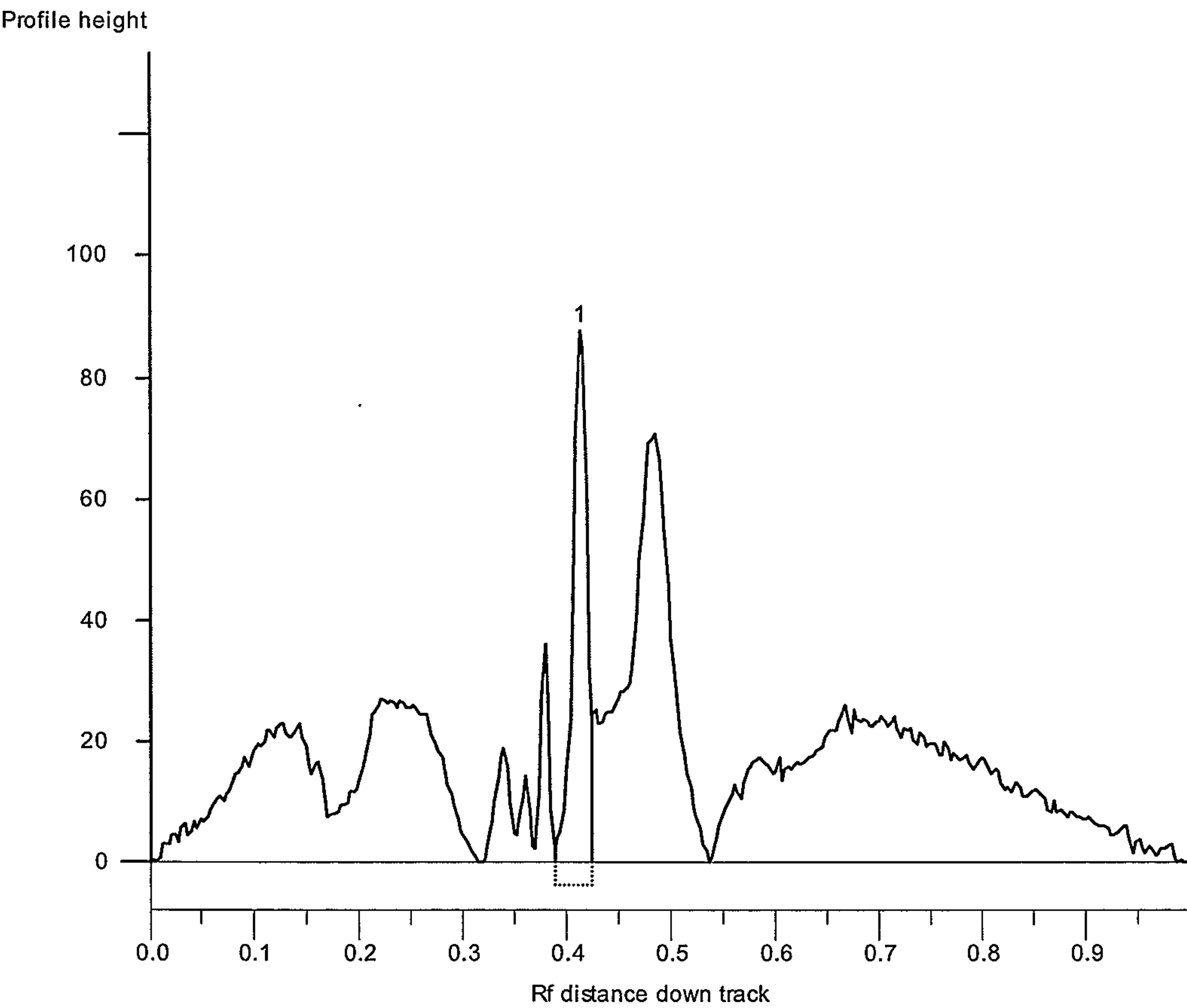
Graph 2

Track 3	Test sample			
	rat1			
Number	Mol. weight	Height	Raw vol.	Quantity
1	0.00	40.132	43488.45	0.65

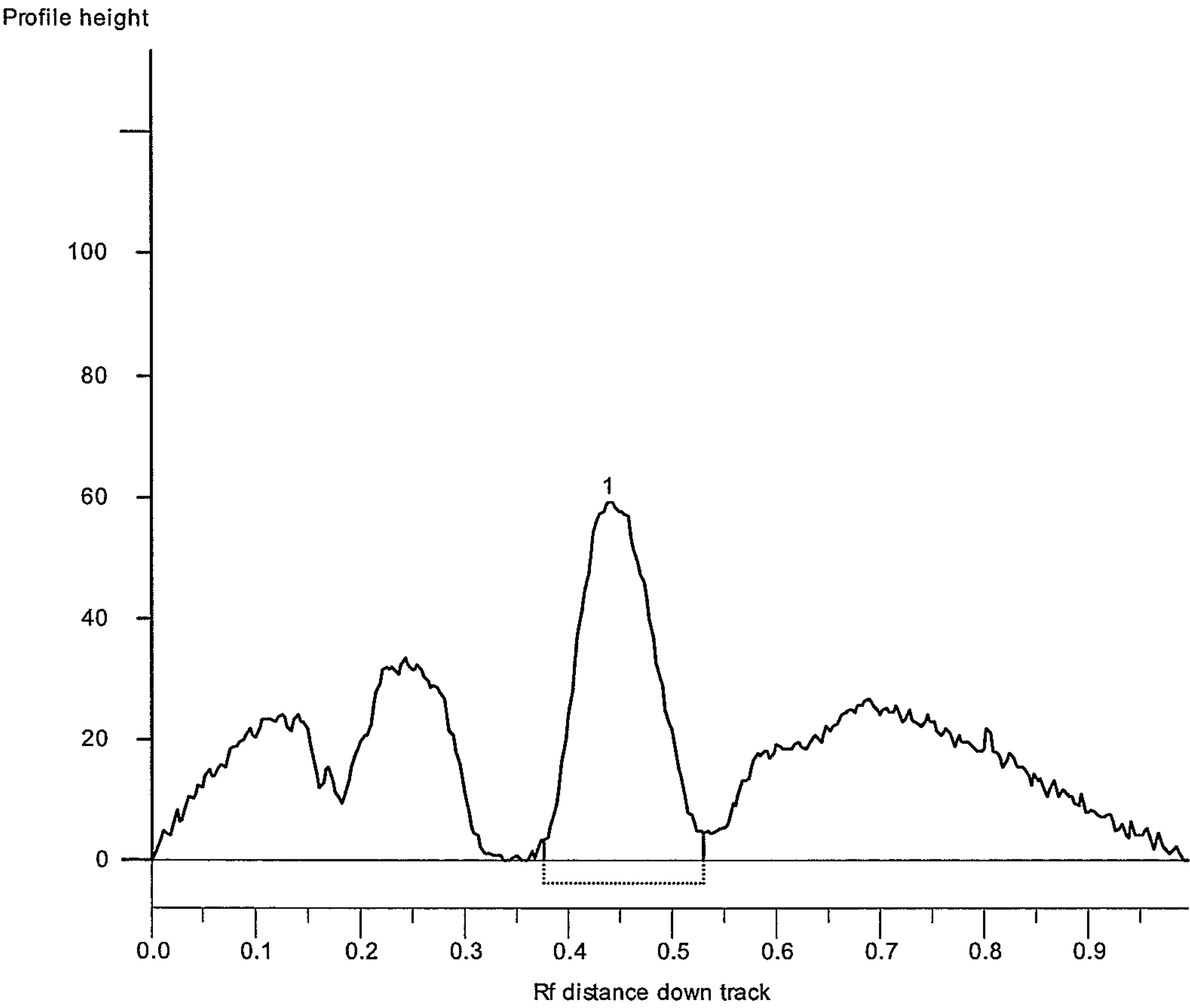


Graph 3

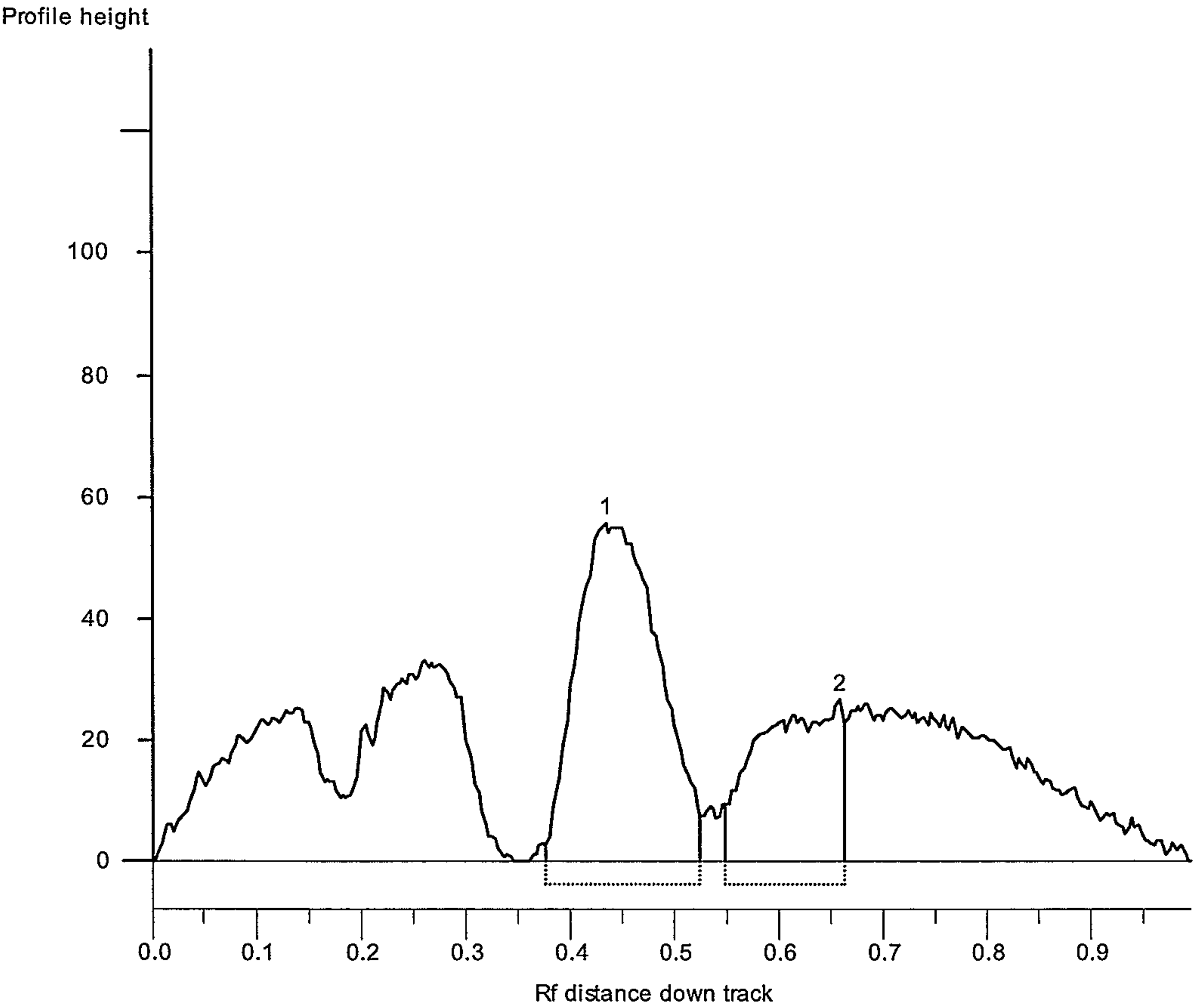
Track 2	Control sample			
	rat 1			
Number	Mol. weight	Height	Raw vol.	Quantity
1	0.00	17.205	1749.95	0.03



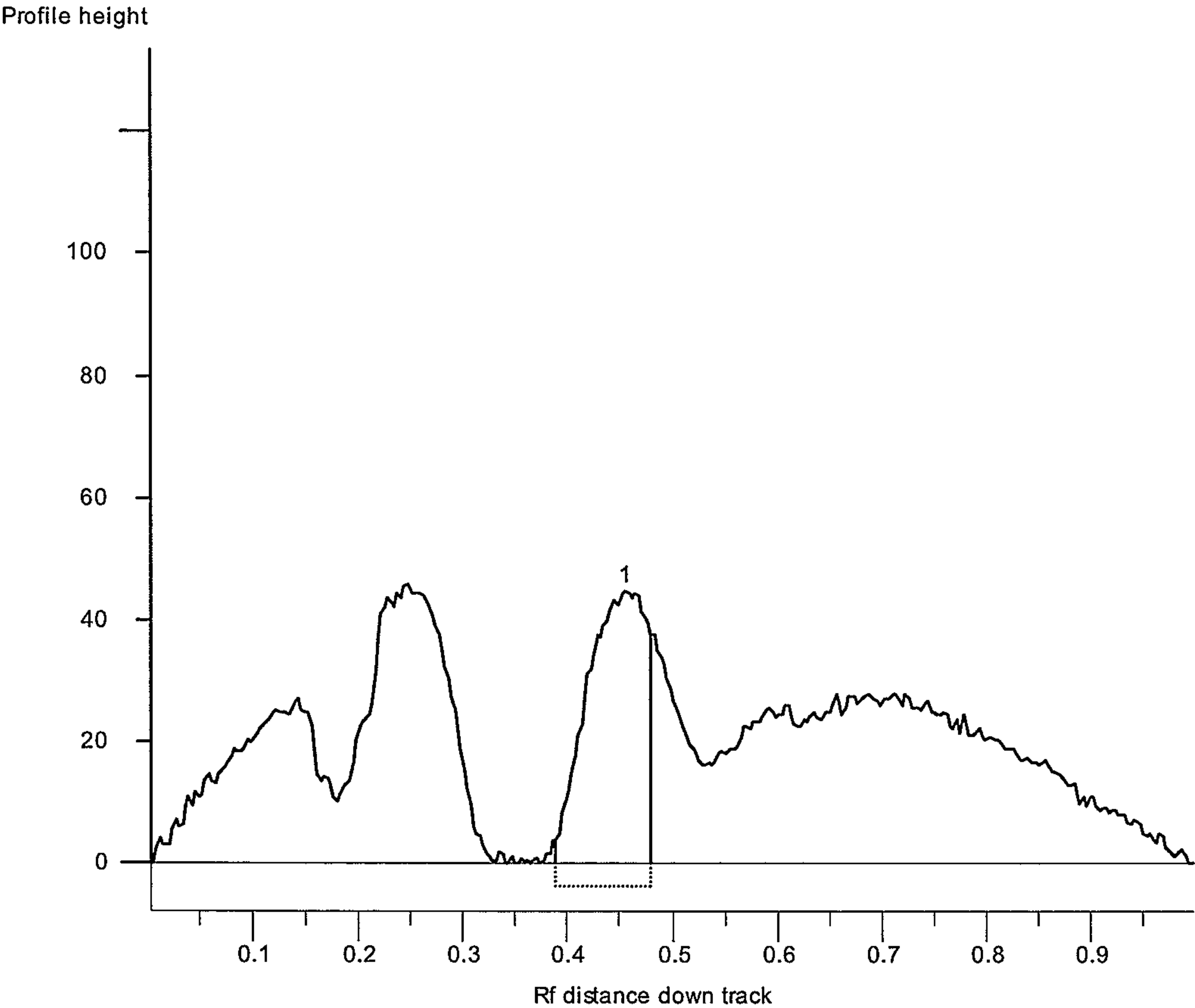
Track 1	Standard peak			
Number	Mol. weight	Height	Raw vol.	Quantity
1	0.00	87.759	22579.82	1.00



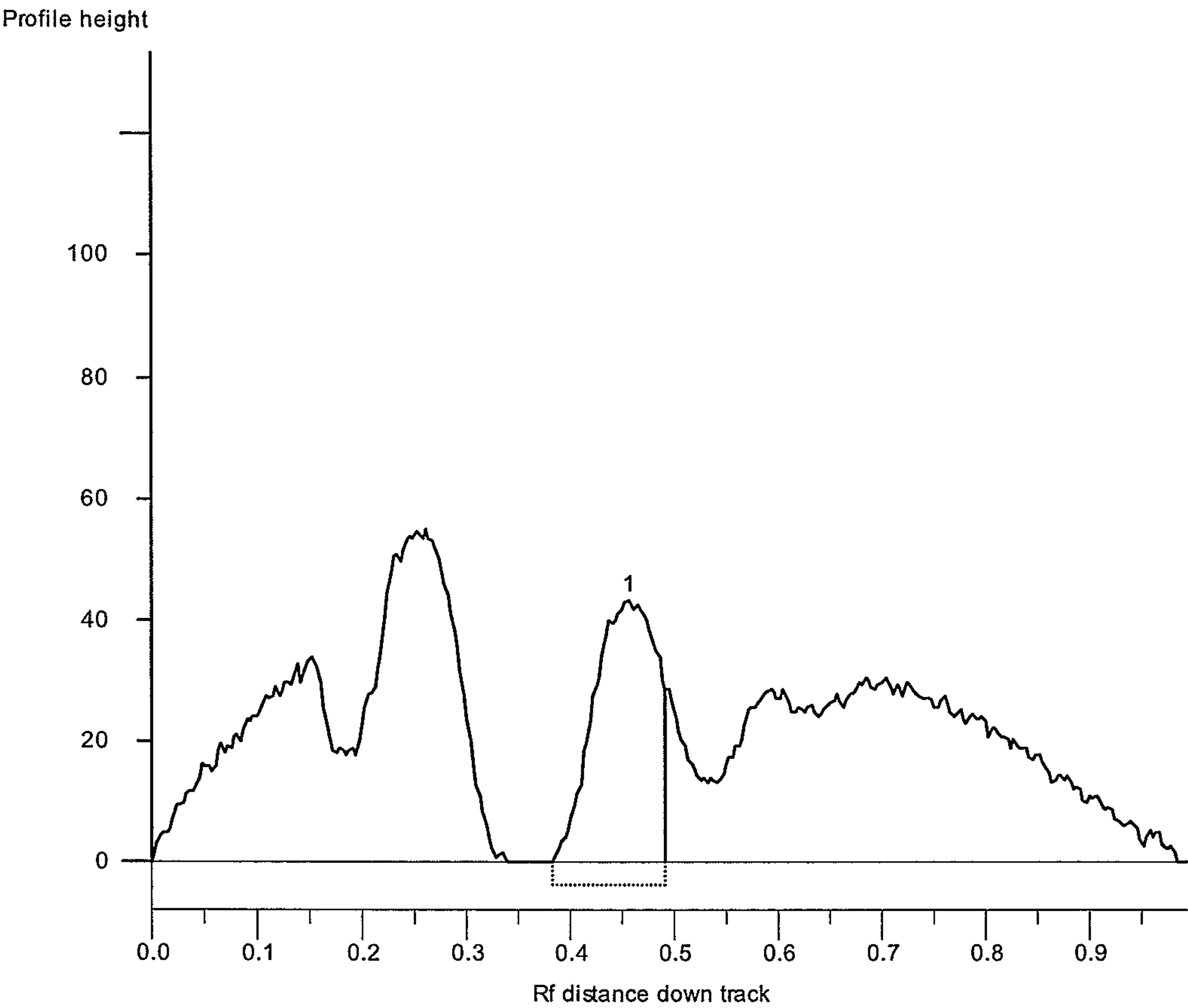
Track 2	Test Rat 2			
Number	Mol. weight	Height	Raw vol.	Quantity
1	0.00	59.209	84890.02	3.76



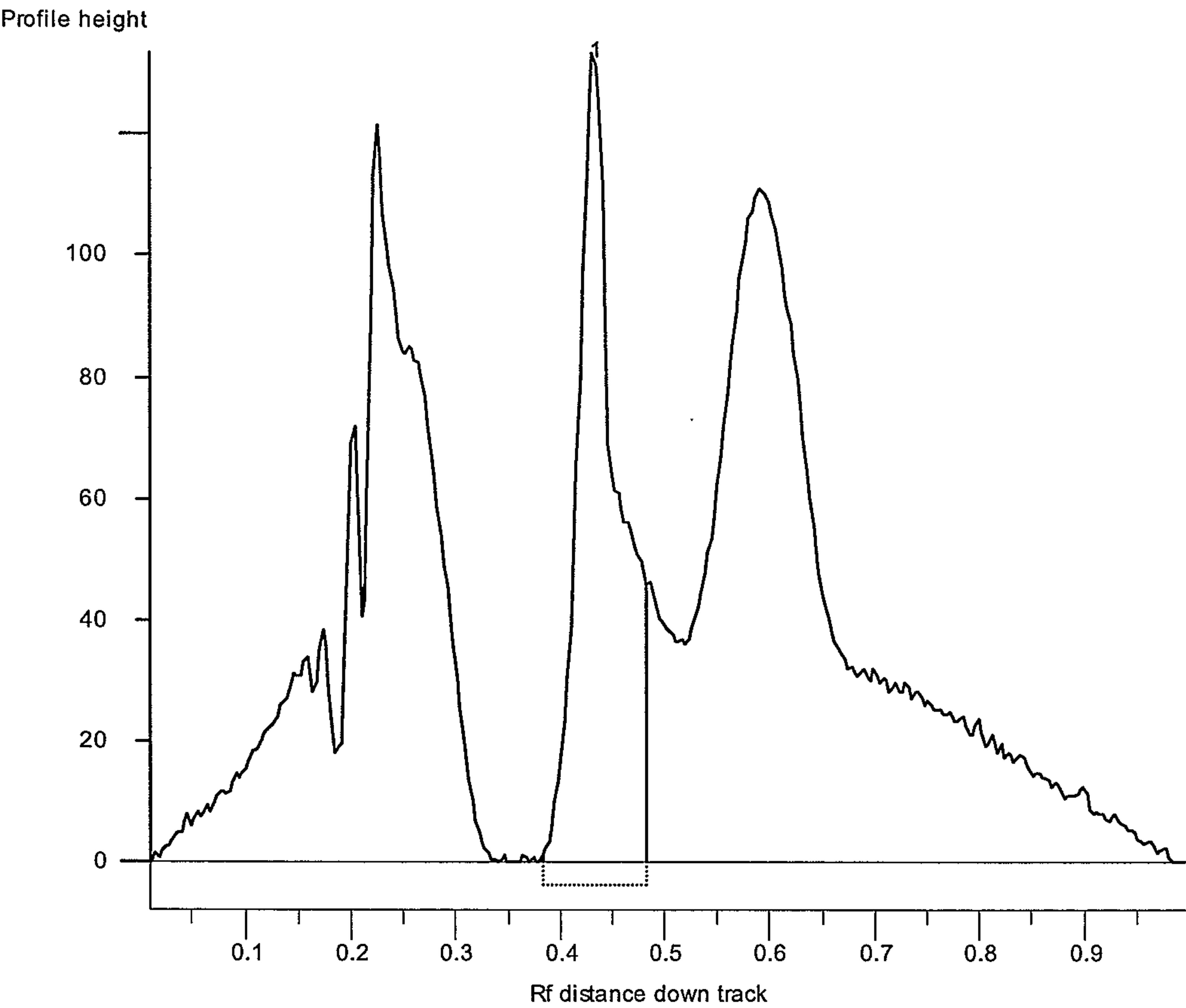
Track 3	Test Rat 3			
Number	Mol. weight	Height	Raw vol.	Quantity
1	0.00	55.640	85474.02	3.79
2	0.00	26.727	39765.56	1.76



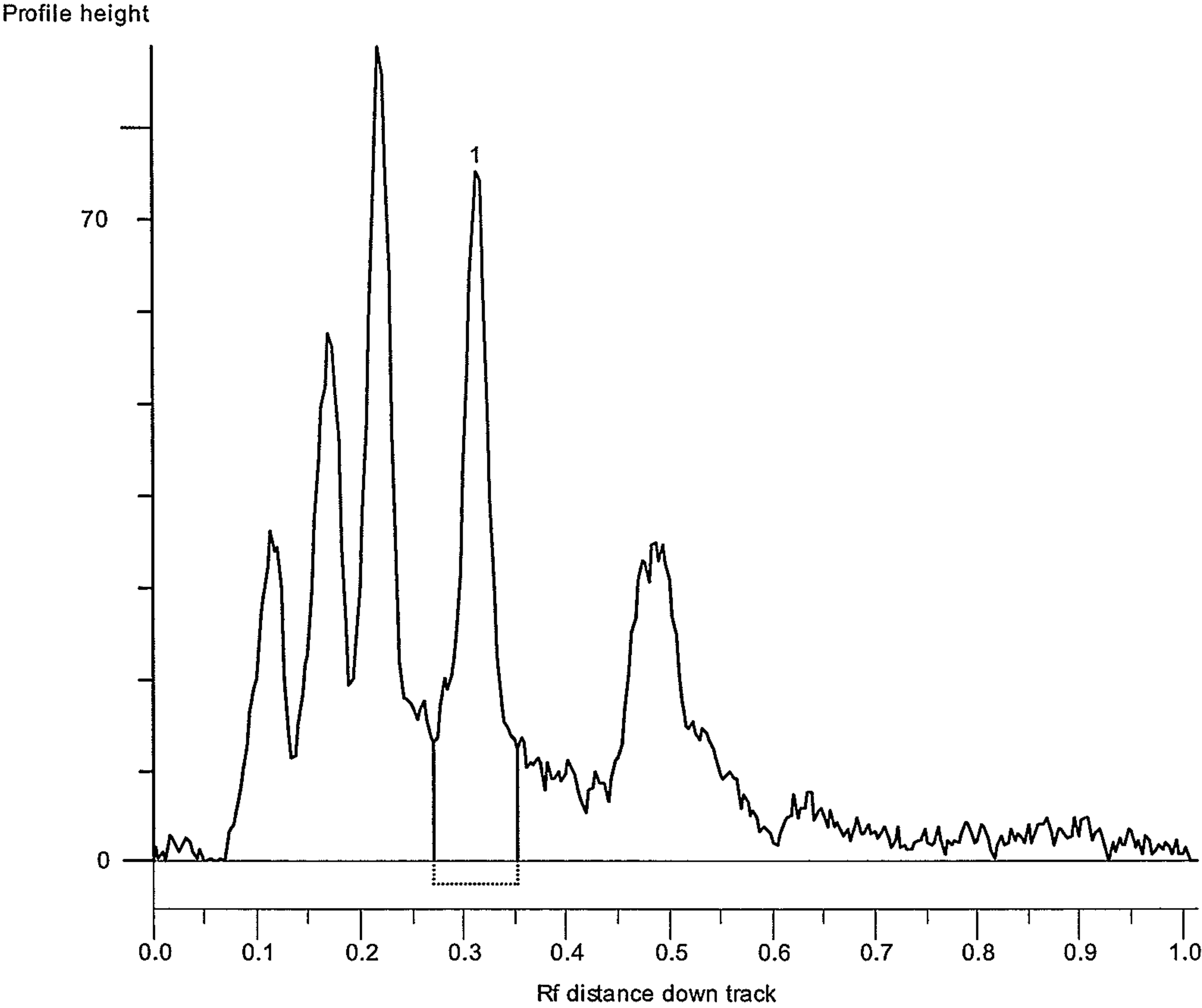
Track 4	Control Rat 2			
Number	Mol. weight	Height	Raw vol.	Quantity
1	0.00	44.873	48653.50	2.15



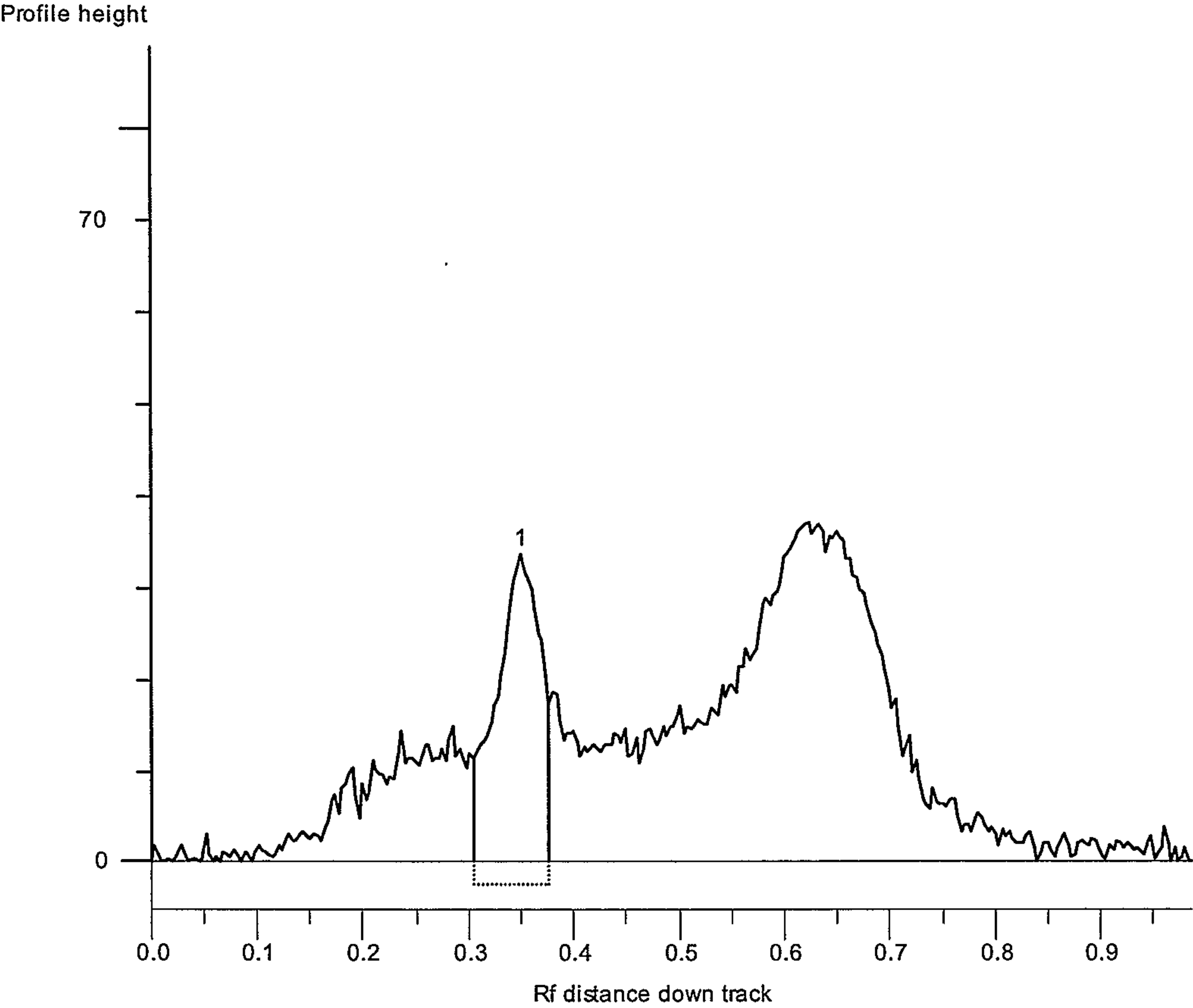
Track 5	Control Rat 3			
Number	Mol. weight	Height	Raw vol.	Quantity
1	0.00	43.340	50354.66	2.23



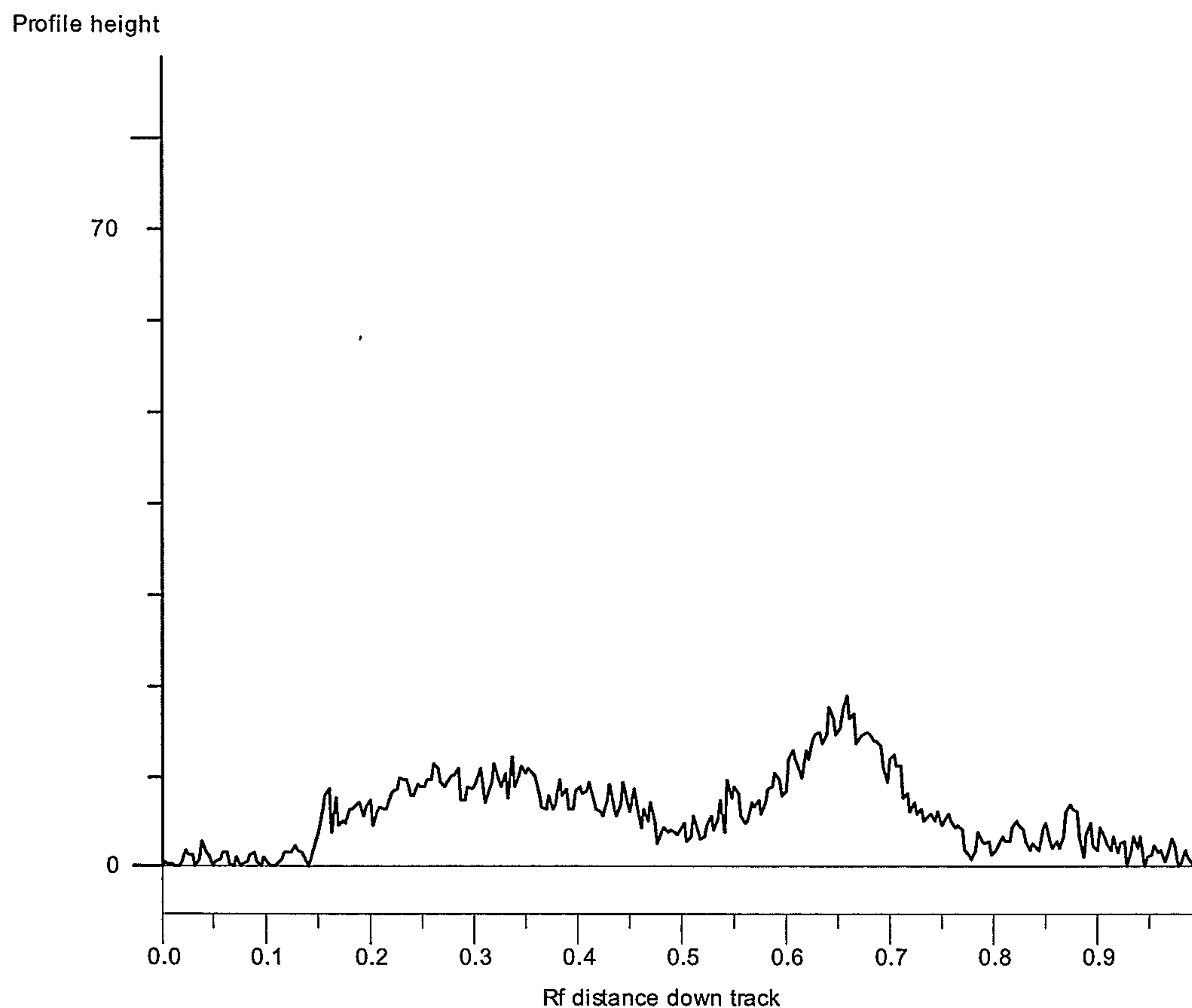
Track 6	UMR106 cells			
Number	Mol. weight	Height	Raw vol.	Quantity
1(m)	0.00	131.463	98700.82	4.37



Track 1	Standard peak			
Number	Mol. weight	Height	Raw vol.	Quantity
1	0.00	75.392	47214.61	1.00



Track 3	Test sample			
	Rat 4			
Number	Mol. weight	Height	Raw vol.	Quantity
1	0.00	33.654	33165.83	0.70



Control Rat 4

No values detectable for this track

9. Limitations of research

The current pilot study has begun to answer some important questions in regards to the signalling controls involved in osteoclast, osteoblast and potentially odontoclastic interactions, there are however many limitations to the study.

The study aims to identify signalling molecules of RANKL and OPG in dental and surrounding tissue that may be important during root resorption. However a major limitation of the research design is that extractive methods of RNA analysis does not

allow the identification of the source of any of the changes seen in the analysis. Does the increase of OPG and RANKL seen in the current study come from similar cells as seen from previous studies ¹⁻³? Crude dissection of bony tissue makes it difficult to discern the exact cells from which the RNA is derived from. Ideally identification should be done using techniques such as *in situ* hybridisation. However this then limits the quantitative analysis as well.

Quantification in the current study design is again at best semi quantitative, and various limitations have been outlined above(Section 6 Quantification of PCR educts) . The researcher would like to see future studies aimed at using techniques like real-time RT-PCR.

Amplification of RANKL in the current study appeared to be more difficult than OPG from the bony tissue. Amplification efficiency appeared to be less efficient. Optimization of a number of primers had already been attempted however, possibly alternate primers may be used in order to attempt to gain better amplification. As alluded to in the manuscript, interpretation of the relatively lower quantities of RANKL need to be examined with caution.

New research has identified the multiple roles of OPG and RANKL and it is clear that bone remodelling and metabolism are not their sole functions. They have important roles in inflammation, immune reactions, lymph node-organogenesis, as wells as mammary gland formation ^{4, 5} Therefore it is highly possible that the change in expression of OPG and RANKL may not be solely involved in control of osteoclastogenesis and it makes good sense that inflammation which is a major component of orthodontic tooth movement and root resorption be a contributory factor in their changes.

Current research has not been able to show useful unique markers of cementocyte and odontoclast function. Identification of cementocyte alone that is separate to osteoclast and

osteoblasts would be ideal in isolating changes that occur only on the root surface and unrelated to bone.

It is clear that much research is still required to assist in the understanding of tooth movement and the pathological processes of root resorption.

10.Future Directions

The discipline in its own laboratories has with previous research established that heavy forces are correlated with increased root resorption (Chan et al 2002). This pilot study has been able to establish a rat model for heavy orthodontic force induced root resorption.

Understanding of the genetic regulation of normal tooth movement, bone and tooth structure remodelling as well as pathological processes would enable clinicians and orthodontist to better understand the cellular and biological events that occur in root resorption during orthodontic treatment. Using an animal model it has allowed us to investigate changes occurring in the local environment of the rat mandible. The rat model can be further used to evaluate other environmental and biological factors that may influence or control the process of root resorption induced by heavy orthodontic forces.

Stemming from the establishment of the current animal model, several projects are already currently underway:

1. The first was to look at the influence of Fluoride intake in rats during tooth development and its effect on the severity of root resorption following orthodontic tooth movement.
2. Literature has established that following orthodontic tooth movement and removal of root substance during root resorption processes, repair of the cementum is readily seen. An extension of the current project is to look at differences in genetic expression following this healing phase
3. Recent literature has identified new cementum proteins “cementum attachment protein” (CAP) ⁶. It was shown that this newly identified protein could be important in adhesion of cementocyte to the cementum. It is hypothesised that

CAP could be significant in the process of root cementum healing following establishment of resorption lacunae, for the attachment and maintenance of the presence of cementocytes and allow formation of new cementum. New investigation is being established to look at the change in expression of CPAP before during and after orthodontic tooth movement.

Various genes have been implicated as associated with changes of bone remodelling including RANKL, OPG, leptin ⁷, connective tissue growth factor/hypertrophic chondrocyte-specific gene product ⁸, Chondromodulin I ⁹, vitamin D receptor ¹⁰.

Recent literature has established a link to certain genes that could be significant to the process of root resorption. There appears to be an implication that genetic makeup of an individual could influence the severity of root resorption. Direct evidence for a genetic component was demonstrated with use of the sib-pair model and estimated the heritability to be 70% ¹¹. Al-Qawasmi et al. ^{12, 13} identified linkage and linkage disequilibrium between the *IL-1B* gene and EARR in orthodontically treated individuals. The same group later identified a link to *TNFRSF11A*, which encodes the receptor activator of nuclear factor kappa B (RANK). The confirmation of such linkage would be helpful clinical information to identify susceptible patients. Analysis of genetic linkage of specific target genes from patients who have had orthodontic treatment and presented with subsequent significant root resorption would confirm the linkage of these target genes. This would confirm the genetic contribution to root resorption process.

Future research aims to increase our understanding of the root resorption process in order to better identify susceptible patients, present more effective treatment planning with minimal potential risks to patients. This would decrease both cost to the community and the individual.

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RANKL AMPLIFICATION FORM TWO RAT MANDIBLES 207

Nucleic acid spectrometry

Beckman DU- 600
Nucleic Acid

Date: 9/9/02

Assay type: General Ratio and Concentration
Units: µg/ml
Read average time: 0.05sec
Dilution factor 50

Sample	Abs 260.0nm	Abs 280.0nm	Bkg abs 320.0nm	<u>260.0</u> 280.0	<u>280.0</u> 260.0	Protein µg/ml	Nucleic Acid µg/ml
1	0.3395	0.2078	0.0136	1.6782	0.5959	54.5688	13.5043
2	0.3555	0.2170	0.0072	1.6599	0.6025	31.8976	14.3526
3	0.2945	0.1797	0.0060	1.6613	0.6019	51.0255	11.8924
4	0.3091	0.1880	0.0030	1.6452	0.6078	57.4529	12.6660
5	0.2116	0.1290	0.0007	1.6442	0.6082	39.3532	8.6461
6	0.2428	0.1512	0.0123	1.6592	0.6026	41.0040	9.4976
7	0.4399	0.2658	0.0016	1.6589	0.6028	56.1498	18.0598
8	0.4944	0.2977	0.0091	1.6816	0.5947	40.3853	20.1376
9	0.5031	0.3026	0.0007	1.6642	0.6009	68.0713	20.7313
10	0.5061	0.3066	0.0139	1.6815	0.5947	81.5395	20.4199
11	1.1557	0.6734	0.0003	1.7166	0.5825	69.6534	48.4464
12	1.1733	0.6835	0.0040	1.7209	0.581	69.0428	49.0858
13	0.04899	0.2944	0.0024	1.6696	0.5990	83.9910	20.1508
14	0.5125	0.3093	0.0119	1.6829	0.5942	82.5607	20.7784
15	0.6319	0.3212	0.0007	1.9697	0.5077	13.3509	28.1665
16	0.7219	0.3697	0.0115	1.9834	0.5042	17.7109	31.7903
17	0.6051	.03368	0.0044	1.7861	0.5599	68.0482	26.0515
18	0.6369	0.3606	0.0153	1.8000	0.5556	65.2339	26.6682
19	0.4105	0.2323	0.0007	1.7647	0.5667	50.2412	17.4766
20	0.4151	0.2337	0.0047	1.7611	0.5678	52.0358	17.8217
21	0.0828	0.0412	0.0054	1.8928	0.5283	5.5262	3.8712
22	0.0874	0.0450	0.0028	1.8885	0.5292	5.8237	3.9555
23	0.1985	0.1113	0.0068	1.8337	0.5454	17.0738	8.2913

Nucleotide information for Rattus Norvegicus GAPDH mRNA

LOCUS AB017801 1242 bp mRNA linear ROD
09-SEP-2000

DEFINITION Rattus norvegicus mRNA for glyceraldehyde-3-phosphate dehydrogenase, complete cds.

ACCESSION AB017801

VERSION AB017801.1 GI:9798637

KEYWORDS glyceraldehyde-3-phosphate dehydrogenase.

SOURCE Rattus norvegicus (Norway rat)

ORGANISM Rattus norvegicus
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Rattus.

REFERENCE 1 (sites)

AUTHORS Tajima,H., Tsuchiya,K., Yamada,M., Kondo,K., Katsube,N. and Ishitani,R.

TITLE Over-expression of GAPDH induces apoptosis in COS-7 cells transfected with cloned GAPDH cDNAs

JOURNAL Neuroreport 10 (10), 2029-2033 (1999)

MEDLINE 99351955

PUBMED 10424669

REFERENCE 2 (bases 1 to 1242)

AUTHORS Tajima,H. and Ishitani,R.

TITLE Direct Submission

JOURNAL Submitted (21-SEP-1998) Hisao Tajima, Ono Pharmaceutical Co., Ltd., Minase Research Institute; 3-1-1 Sakurai, Shimamoto, Osaka 618-8585, Japan (E-mail:tajima@ono.co.jp, Tel:075-961-1151, Fax:075-962-9314)

FEATURES Location/Qualifiers

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/db_xref="taxon:10116"
/cell_type="primary cultured cerebellar granule cells"

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IPELNGKLTGMAFRVPTPNVSVVDLTCRLEKPAKYDDIKKVVKQAAEGPLKGILGYTE

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BASE COUNT 295 a 364 c 318 g 265 t

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Nucleotide information for Rattus Norvegicus OPG mRNA

LOCUS RNU94330 2432 bp mRNA linear ROD
05-MAY-1997
DEFINITION Rattus norvegicus osteoprotegerin (OPG) mRNA, complete cds.
ACCESSION U94330
VERSION U94330.1 GI:2072180
KEYWORDS .
SOURCE Rattus norvegicus (Norway rat)
ORGANISM Rattus norvegicus
Euteleostomi; Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Rattus.
REFERENCE 1 (bases 1 to 2432)
AUTHORS Simonet,W.S., Lacey,D.L., Dunstan,C.R., Kelley,M., Chang,M.S., Luthy,R., Nguyen,H.Q., Wooden,S., Bennett,L., Boone,T., Shimamoto,G., DeRose,M., Elliott,R., Colombero,A., Tan,H.L., Trail,G., Sullivan,J., Davy,E., Bucay,N., Renshaw-Gegg,L., Hughes,T.M., Hill,D., Pattison,W., Campbell,P., Sander,S., Van,G., Tarpley,J., Derby,P., Lee,R., Amgen EST Program and Boyle,W.J.
TITLE Osteoprotegerin: a novel secreted protein involved in the regulation of bone density
JOURNAL Cell 89 (2), 309-319 (1997)
MEDLINE 97262071
PUBMED 9108485
REFERENCE 2 (bases 1 to 2432)
AUTHORS Boyle,W.J.
TITLE Direct Submission
JOURNAL Submitted (18-MAR-1997) Department of Cell Biology, Amgen, Inc., 1840 Dehavilland Drive, Thousand Oaks, CA 91320, USA
FEATURES Location/Qualifiers
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RKHTNCSSLGLLLIQKGNATHDNCVCSGNREATQNCGIDVTLC EEAFFRFAVPTKIIPN
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BASE COUNT 682 a 564 c 562 g 624 t
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Nucleotide information for Rattus Norvegicus RANKL mRNA

LOCUS NM_057149 957 bp mRNA linear ROD
06-APR-2003
DEFINITION Rattus norvegicus tumor necrosis factor (ligand) superfamily, member 11 (Tnfsf11), mRNA.
ACCESSION NM_057149
VERSION NM_057149.1 GI:16924011
KEYWORDS .
SOURCE Rattus norvegicus (Norway rat)
ORGANISM Rattus norvegicus
Euteleostomi; Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Rattus.
REFERENCE 1 (bases 1 to 957)
AUTHORS Odgren,P.R., Kim,N., van Wesenbeeck,L., MacKay,C., Mason-Savas,A., Safadi,F.F., Popoff,S.N., Lengner,C., van-Hul,W., Choi,Y. and Marks,S.C. Jr.
TITLE Evidence that the rat osteopetrotic mutation toothless (tl) is not in the TNFSF11 (TRANCE, RANKL, ODF, OPGL) gene
JOURNAL Int. J. Dev. Biol. 45 (8), 853-859 (2001)
MEDLINE 21662371
PUBMED 11804028
REMARK GeneRIF: the toothless (tl) osteopetrotic rat mutation is not in the TNFSF11 locus
REFERENCE 2 (bases 1 to 957)
AUTHORS Xu,J., Tan,J.W., Huang,L., Gao,X.H., Laird,R., Liu,D., Wysocki,S. and Zheng,M.H.
TITLE Cloning, sequencing, and functional characterization of the rat homologue of receptor activator of NF-kappaB ligand
JOURNAL J. Bone Miner. Res. 15 (11), 2178-2186 (2000)
MEDLINE 20540945
PUBMED 11092398
COMMENT PROVISIONAL REFSEQ: This record has not yet been subject to final NCBI review. The reference sequence was derived from AF187319.1.
FEATURES
source Location/Qualifiers
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family. Family
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complexes. TNF mediates mature T-cell receptor-
apoptosis through the p75 TNF receptor"
/ db_xref="CDD:smart00207"
BASE COUNT 230 a 260 c 256 g 211 t
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ggctccagcg
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UMR 106 cell lines were used as control for expression of GAPDH, OPG and RANKL

UMR 106 are *Rattus norvegicus* derived cell line that is known to express the genes of interest

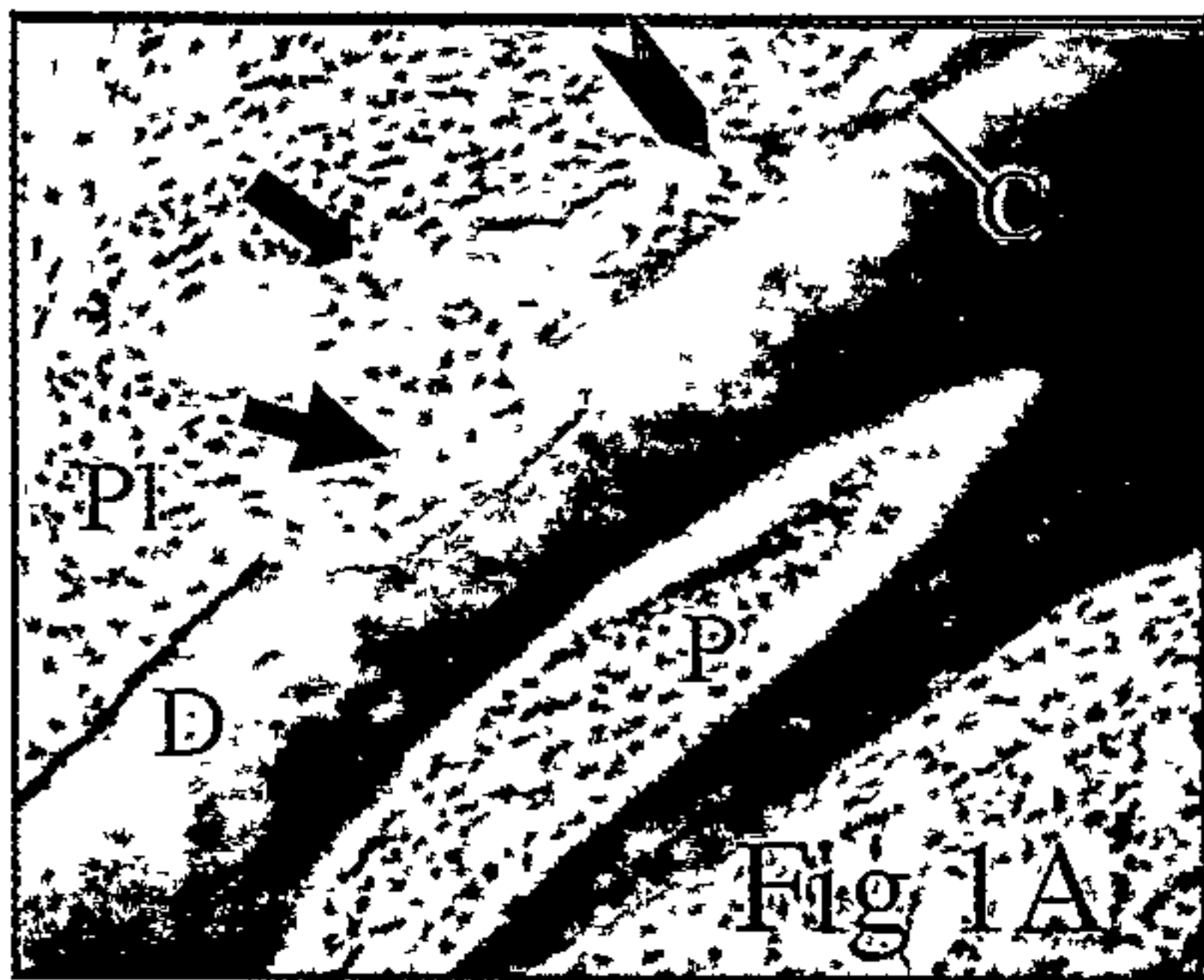
From American Type Culture Collection

Cell Lines	
ATCC Number:	CRL-1661 Order this item
Price:	\$215.00
Designation:	UMR-106
Depositors:	AE Bogden
Biosafety Level:	1
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	<i>Rattus norvegicus</i> (rat) deposited as <i>Rattus</i> sp.
Morphology:	epithelial
Tissue:	bone; osteosarcoma
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Related Cell Culture Products	
Comments:	The UMR-106 cell line is a clonal derivative of a transplantable rat osteosarcoma that had been induced by injection of radiophosphorous (32P). The cells are responsive to PTH, prostaglandins and bone resorbing steroids. The PTH responsiveness of UMR-106 is greater than that of the related cell line UMR-108 (ATCC CRL-1663). Activation of protein kinase C inhibits ATP induced increases in intracellular calcium levels. Both the original sarcoma and the cloned line were developed by T.J. Martin at the University of Sheffield.
Receptors Expressed:	parathyroid hormone (PTH); 1-25(OH)2D3 (bone resorbing steroid hormone)
Strain:	Sprague-Dawley
Propagation:	ATCC medium: Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, 90%; fetal bovine serum, 10% Temperature: 37.0 C
Subculturing:	Remove medium, and rinse with 0.25% trypsin, 0.03% EDTA solution. Remove the solution and add an additional 1 to 2 ml of trypsin-EDTA solution. Allow the flask to sit at room temperature (or at 37C) until the cells detach. Add fresh culture medium, aspirate and dispense into new culture flasks.
Split Ratio:	A subcultivation ratio of 1:4 to 1:8 is recommended
Fluid Renewal:	2 to 3 times per week
Freeze Medium:	culture medium 95%; DMSO, 5%

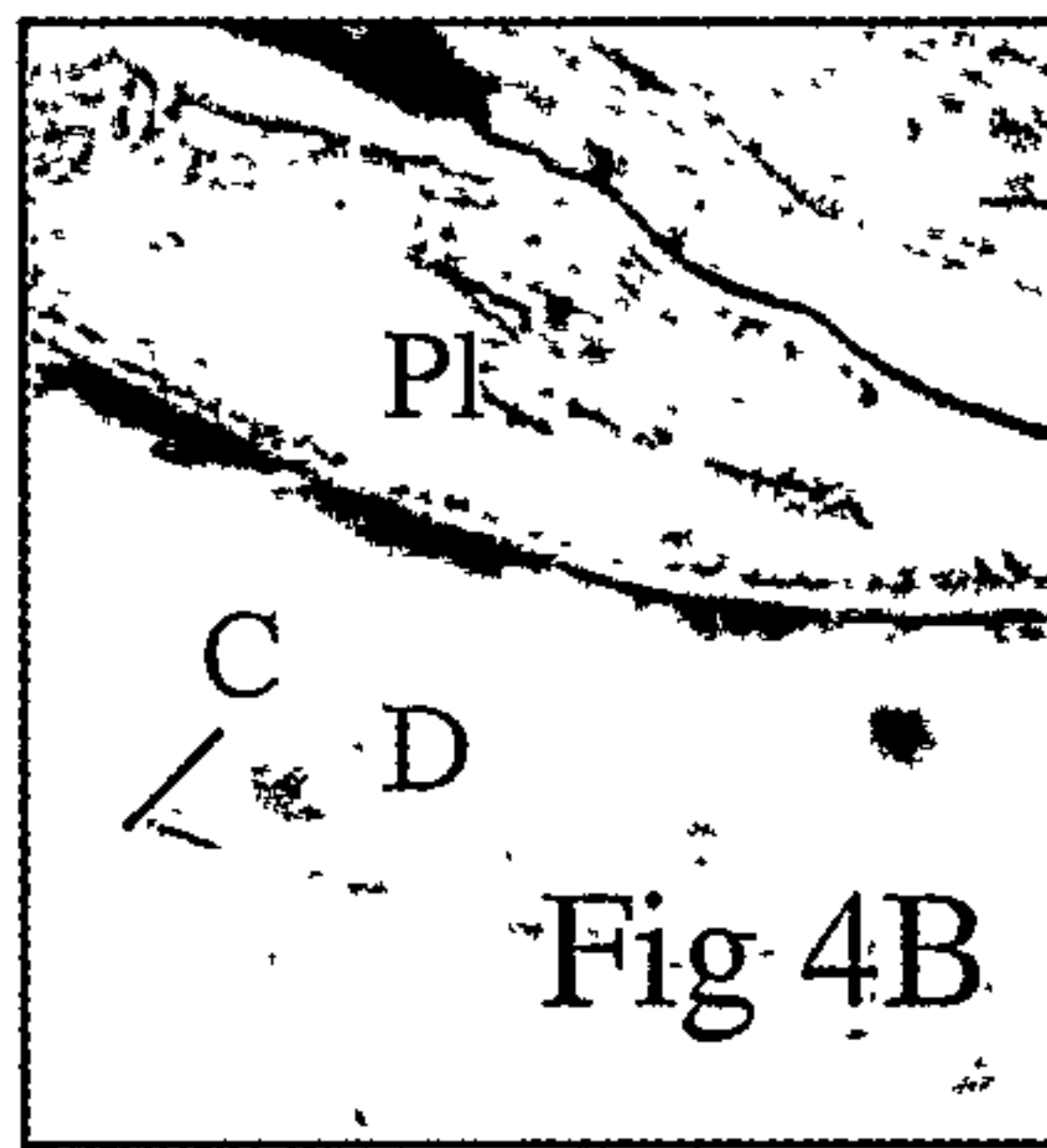
Related Products:	Recommended medium (without the additional supplements or serum described under ATCC Medium) - ATCC No: 30-2002 recommended serum - ATCC No: 30-2020
References:	21441: Banerjee C , et al. An AML-1 consensus sequence binds an osteoblast-specific complex and transcriptionally activates the osteoclastin gene. Proc. Natl. Acad. Sci. USA 93: 4968-4973, 1996. PubMed: 8643513 21887: Copp, D.H.; Talmage, R.V., eds. , editor. Endocrinology of calcium metabolism. Amsterdam: Elsevier/North Holland; 1977, pp. 264-269. 22331: Atkins D , et al. Rat osteogenic sarcoma cells: modulation of hormone stimulated cyclic AMP production by prostaglandin antagonists and biosynthesis inhibitors. Clin. Exp. Pharmacol. Physiol. 7: 31-44, 1980. PubMed: 6103768 22684: Manolagas SC , Deftos LJ . Cytoreceptor assay for 1,25-dihydroxyvitamin D3: a novel radiometric method based on binding of the hormone to intracellular receptors in vitro. Lancet 2: 401-402, 1980. PubMed: 6105521 22771: Partridge NC , et al. Receptors for 1,25(OH)2-vitamin D3 enriched in cloned osteoblast-like rat osteogenic sarcoma cells. FEBS Lett. 115: 139-142, 1980. PubMed: 6248375 22847: Ingleton PM , et al. Alkaline phosphatase in serum and tumour of rats bearing a hormone- responsive transplantable osteogenic sarcoma. Eur. J. Cancer 15: 685-691, 1979. PubMed: 292595 22873: Crawford A , et al. Membranes from a transplantable osteogenic sarcoma responsive to parathyroid hormone and prostaglandins: regulation of adenylate cyclase and of hormone metabolism. J. Endocrinol. 77: 213-224, 1978. PubMed: 275436 22874: Atkins D , Martin TJ . Rat osteogenic sarcoma cells:effects of some prostaglandins, their metabolites and analogues on cyclic AMP production. Prostaglandins 13: 861-871, 1977. PubMed: 194286 22898: Martin TJ , et al. Parathyroid hormone-responsive adenylate cyclase in induced transplantable osteogenic rat sarcoma. Nature 260: 436-438, 1976. PubMed: 1062678 22974: Ingleton PM , et al. Radiation induced osteogenic sarcoma in the rat as a model of hormone-responsive differentiated cancer. Lab. Anim. Sci. 27: 748-756, 1977. PubMed: 338978 22975: Atkins D , et al. Rat osteogenic sarcoma cells: isolation and effects of hormones on the production of cyclic AMP and cyclic GMP. Endocrinology 101: 555-562, 1977. PubMed: 195798 22989: Crawford A , et al. Rat osteogenic sarcoma cells: comparison of the effects of prostaglandins E1, E2, I2 (prostacyclin), 6-keto F1alpha and thromboxane B2 on cyclic AMP production and adenylate cyclase activity. Biochem. Biophys. Res. Commun. 82: 1195-1201, 1978. PubMed: 212039 23013: Gallinaro BJ , et al. Activation of protein kinase C inhibits ATP-induced [Ca2+]i elevation in rat osteoblastic cells: selective effects on P2Y and P2U signaling pathways. J. Cell. Physiol. 162: 305-314, 1995. PubMed: 7860638 23381: Clin. Orthop. Relat. Res. 140: 247-254, 1979. 23398: Manolagas SC , et al. 1,25-Dihydroxyvitamin D3 receptor-like macromolecule in rat osteogenic sarcoma cell lines. J. Biol. Chem. 255: 4414-4417, 1980. PubMed: 6929275 23491: Underwood JC , et al. Structural and functional correlations in parathyroid hormone responsive transplantable osteogenic sarcomas. Eur. J. Cancer 15: 1151-1158, 1979. PubMed: 294355 24392: Trans. Biochem. Sic. 6: 239-241, 1978. 26135: Partridge NC , et al. Morphological and biochemical characterization of four clonal osteogenic sarcoma cell lines of rat origin. Cancer Res. 43: 4308-4315, 1983. PubMed: 6575864 26136: Martin TJ , et al. Hormonal influences on bone cells. Methods Enzymol. 145: 324-336, 1987. PubMed: 3474491 26137: Forrest SM , et al. Characterization of an osteoblast-like clonal cell line which responds to both parathyroid hormone and calcitonin. Calcif. Tissue Int. 37: 51-56, 1985. PubMed: 3922597 33152: Hocking AM , et al. Eukaryotic expression of recombinant biglycan. J. Biol. Chem. 271: 19571-19577, 1996. PubMed: 8702651

Haematoxylin and Eosin stained paraffin sections of test and control rat mandibles to confirm histologically presence of root resorption

Paraffin sections of the mesial surface of a tooth subjected to orthodontic forces (A) and a control tooth (B). The normal structure of the tooth and periodontal ligament was readily recognized, including pulp (P), dentine (D), cementum (C), the periodontal ligament (PL) and bone (Bn). Occasional large vessels (V) were noted entering the periodontal ligament from the adjacent alveolar bone in both control and test tissues. In teeth subjected to orthodontic forces, resorption lacunae were noted, often eroding through the cementum into the dentine (arrows) while a chronic inflammatory infiltrate and multinucleated giant cells (arrow heads) were also present, although occasionally not within lacunae (red arrows). These observations are consistent with the induction of root resorption by multinucleated giant cells in teeth subjected to orthodontic forces. (H&E, Bars = 100 μ m)



H & E of root
resorption
process (Fig 1
& 2)



H & E of root on
control side

Primers tested

Primers were designed on “Amplify program” (Apple Macintosh)
Primers that were finally chosen to be used in semi quantitative analysis are listed in
Table 1 in Manuscript (section 8)
Base pairs (bp)

Gene	Forward primer	Reverse primer	bp
OPG	TCCTGGCACCTACCTAAAACAGCA	CTACACTCTCGGCATTCACTTTGG	578
RANKL1	CACACCTCACCATCAATGCTGC	GAAGGGTTGGACACCTGAATGC	394
RANKL2	ACGCAGATTTGCAGGACTCGAC	TTCGTGCTCCCTCCTTTCATC	492
GAPDH1	GGTCGGTGTGAACGGATTTGG	ATGTAGGCCATGAGGTCCACC	983
GAPDH2	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	545

Optimization of GAPDH amplification

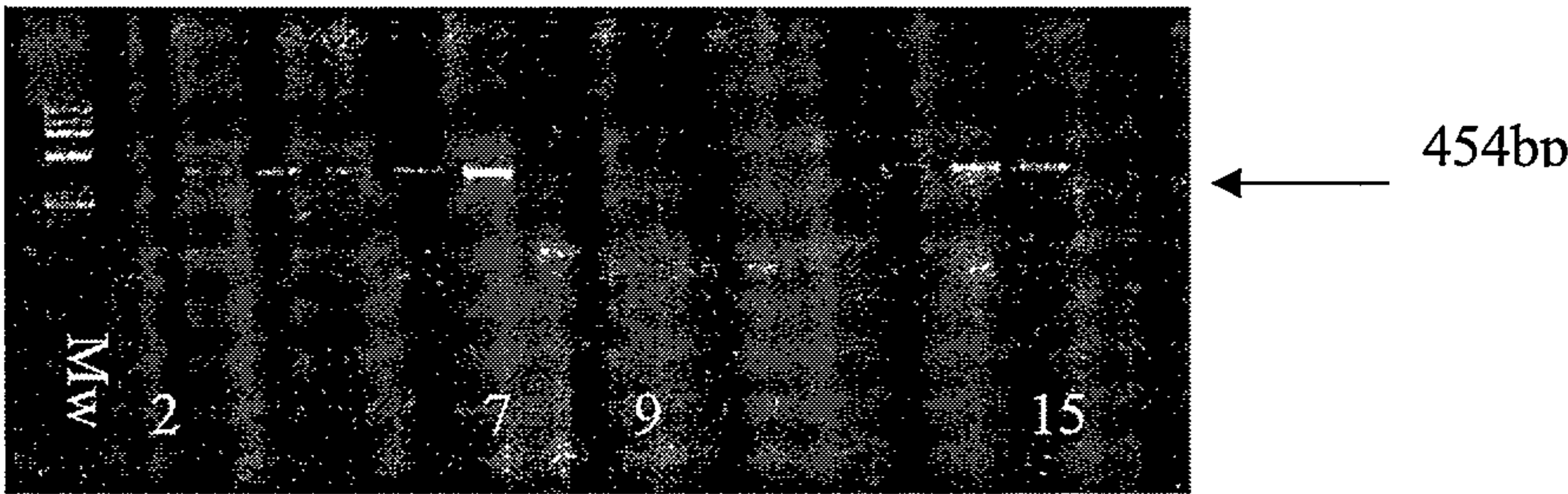
Figure1 shows Agarose gel electrophoresis of RT-PCR product of GAPDH for optimization of PCR

Figure

Lane 1 is molecular weight marker IX (Roche)

Lane 2-7 – optimization with different MgCl2 concentrations at 55°C annealing temperature

9-14 optimization with different MgCl2 concentrations at 60 °C annealing temperature



9-15

Optimization of OPG amplification from control RNA (UMR 106 cells)

Figure 2 shows Agarose gel electrophoresis of RT-PCR product of OPG for optimization of PCR

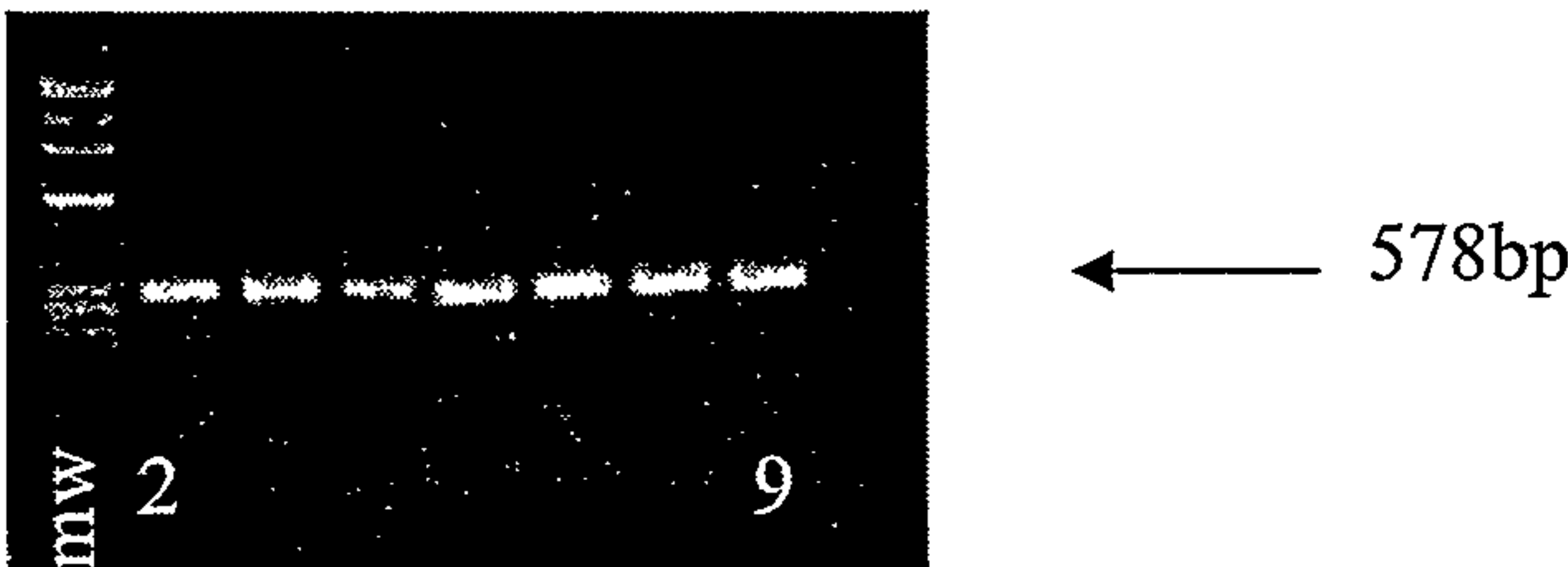
Lane 1 is molecular weight marker IX (Roche)

Lane 2- 9 RT-PCR amplification of OPG from control cells UMR106 cells using different increasing number of cycles

Lane 2 :25 cycles used and increasing 5 cycles for each subsequent lane

Lane 9: 55 cycles used

Band shown at 578bp



Semiquantitative PCR of OPG expression on Test samples

Figure shows agarose gel electrophoresis of GAPDH and OPG for the analysis of expression levels in test samples

Gel A

Lane 1 is molecular weight marker IX

Lane 2 is amplification of control mRNA

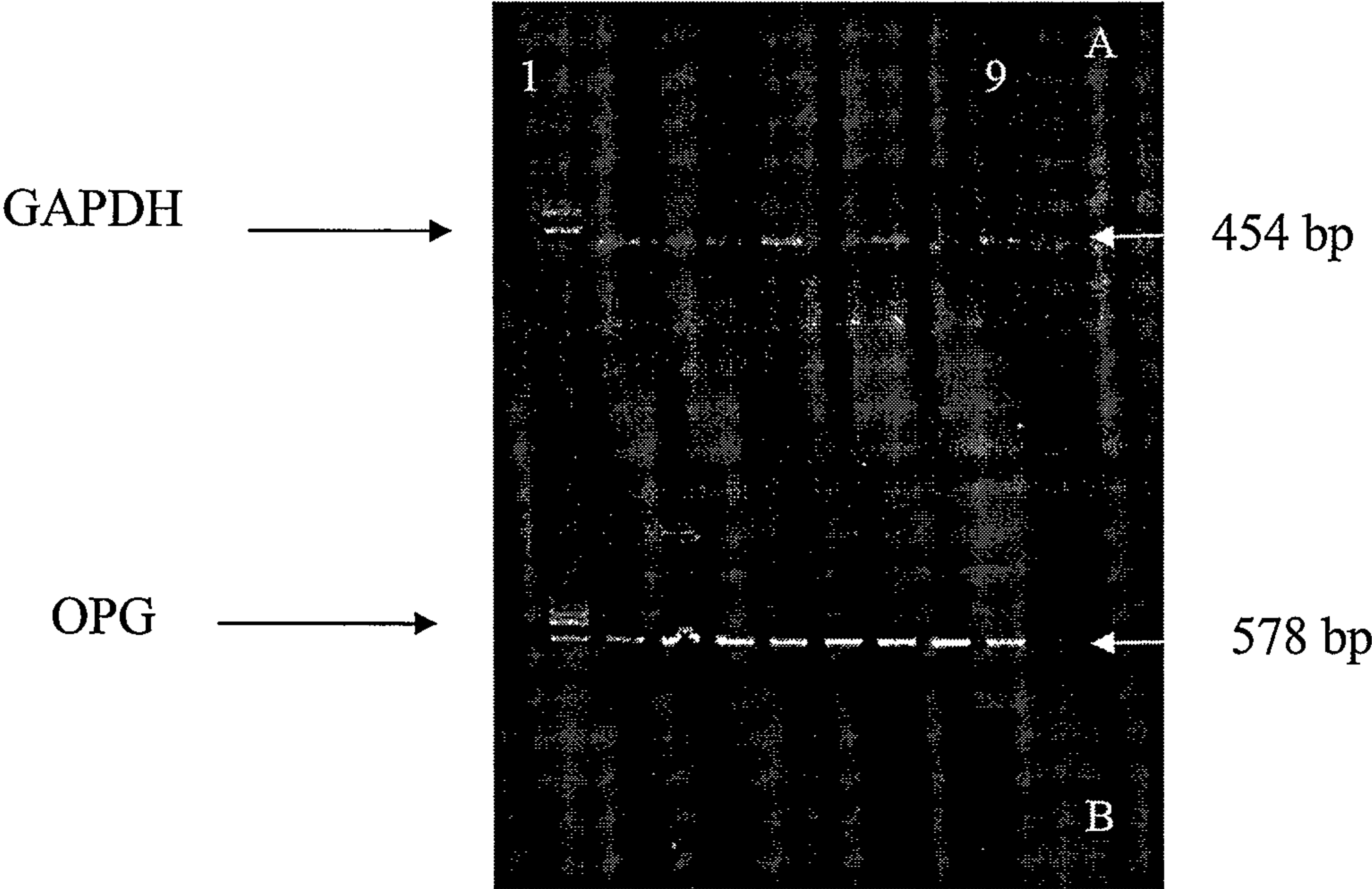
Lane 3-9 test sample mRNA amplified for GAPDH

Gel B

Lane 1 is molecular weight marker IX

Lane 2 is amplification of control mRNA

Lane 3-9 test sample mRNA amplified for OPG



Semiquantitative PCR of OPG expression on Control samples

Figure shows agarose gel electrophoresis of GAPDH and OPG for the analysis of expression levels in control samples

Gel A

Lane 1 is molecular weight marker IX

Lane 2 is amplification of control mRNA

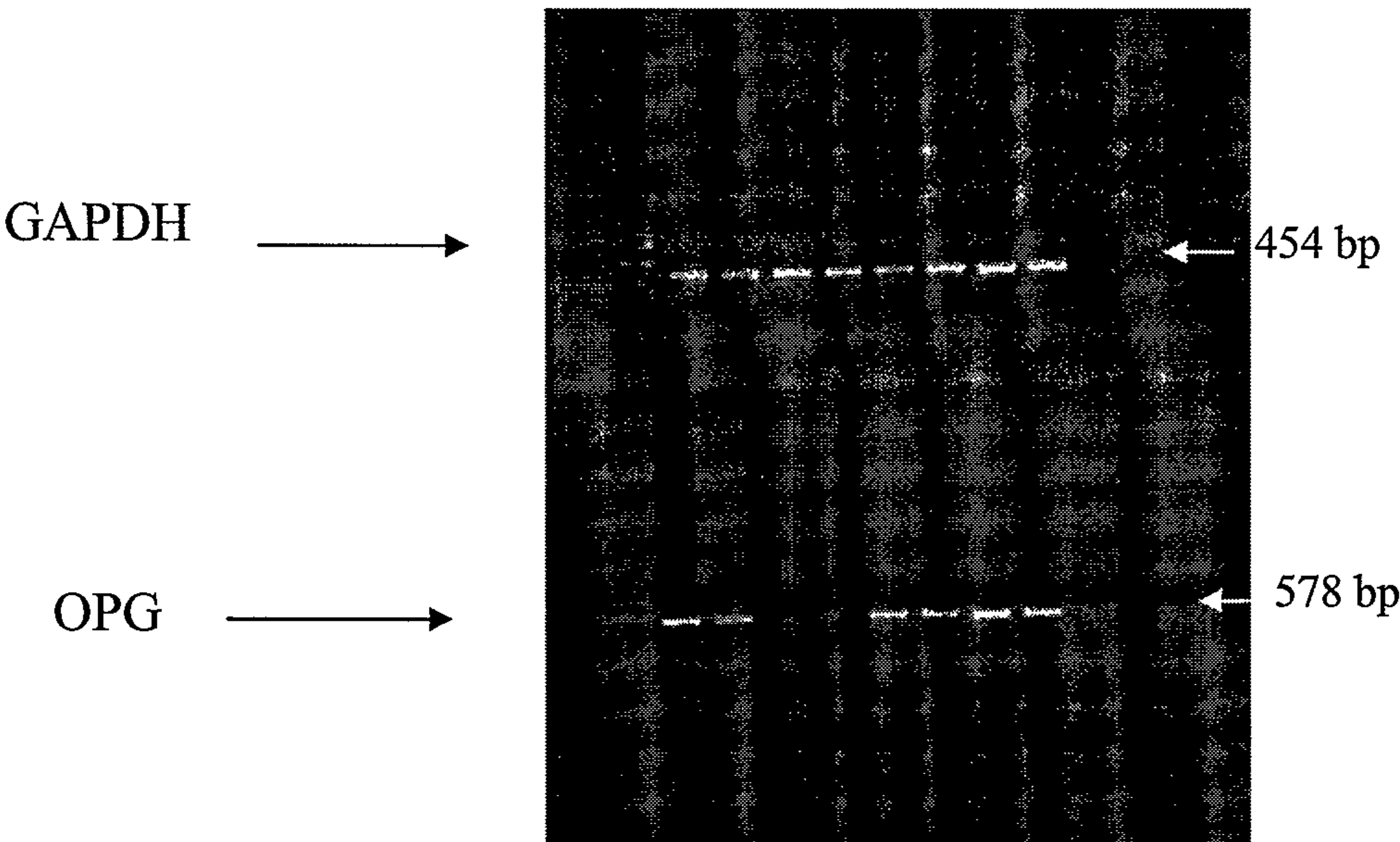
Lane 3-9 test sample mRNA amplified for GAPDH

Gel B

Lane 1 is molecular weight marker IX

Lane 2 is amplification of control mRNA

Lane 3-9 test sample mRNA amplified for OPG



GelDoc analysis of OPG gene expression from test (left) side of rat mandibles

Description							
Track 1		Molecular weight marker					
Number			Height	Raw vol.	Quantity	Ratios of GAPDH/OPG	Ratios of OPG/ GAPDH
1		0	12727.18	3127450	1		
2							
Track 2		Positive control from UMR106 Cells					
Number			Height	Raw vol.	Quantity		
1		GAPDH	12561.63	6420497	2.052949		
2		OPG	16431.63	5200813	1.662956	1.3	0.7
Track 3							
Number			Height	Raw vol.	Quantity		
1		GAPDH	10177.25	5439818	1.739378		
2		OPG	24325.32	8497687	2.71713	0.6	1.67
Track 4							
Number			Height	Raw vol.	Quantity		
1		GAPDH	12019.88	6063816	1.938901		
2		OPG	38371.83	8576761	2.742414	0.7	1.4
Track 5							
Number			Height	Raw vol.	Quantity		
1		GAPDH	15081.35	6673528	2.133856		
2		OPG	25833.43	6744681	2.156607	1	1
Track 6							
Number			Height	Raw vol.	Quantity		
1		GAPDH	4651.051	1626951	0.520217	0.2	5
2		OPG	26728.48	6645095	2.124764		
Track 7							
Number			Height	Raw vol.	Quantity		
1		GAPDH	10745.58	5216899	1.6681		
2		OPG	29896.44	7433088	2.376725	0.7	1.4
Track 8							
Number			Height	Raw vol.	Quantity		
1		GAPDH	4263.139	2011672	0.643231		
2		OPG	39916.82	9699384	3.101371	0.2	5
Track 9							
Number			Height	Raw vol.	Quantity		

Track 10	1	0	11849.88	5922525	1.893723		
	2	0	27194.49	7109007	2.2731	0.8	1.25
	Negative control no expression seen						

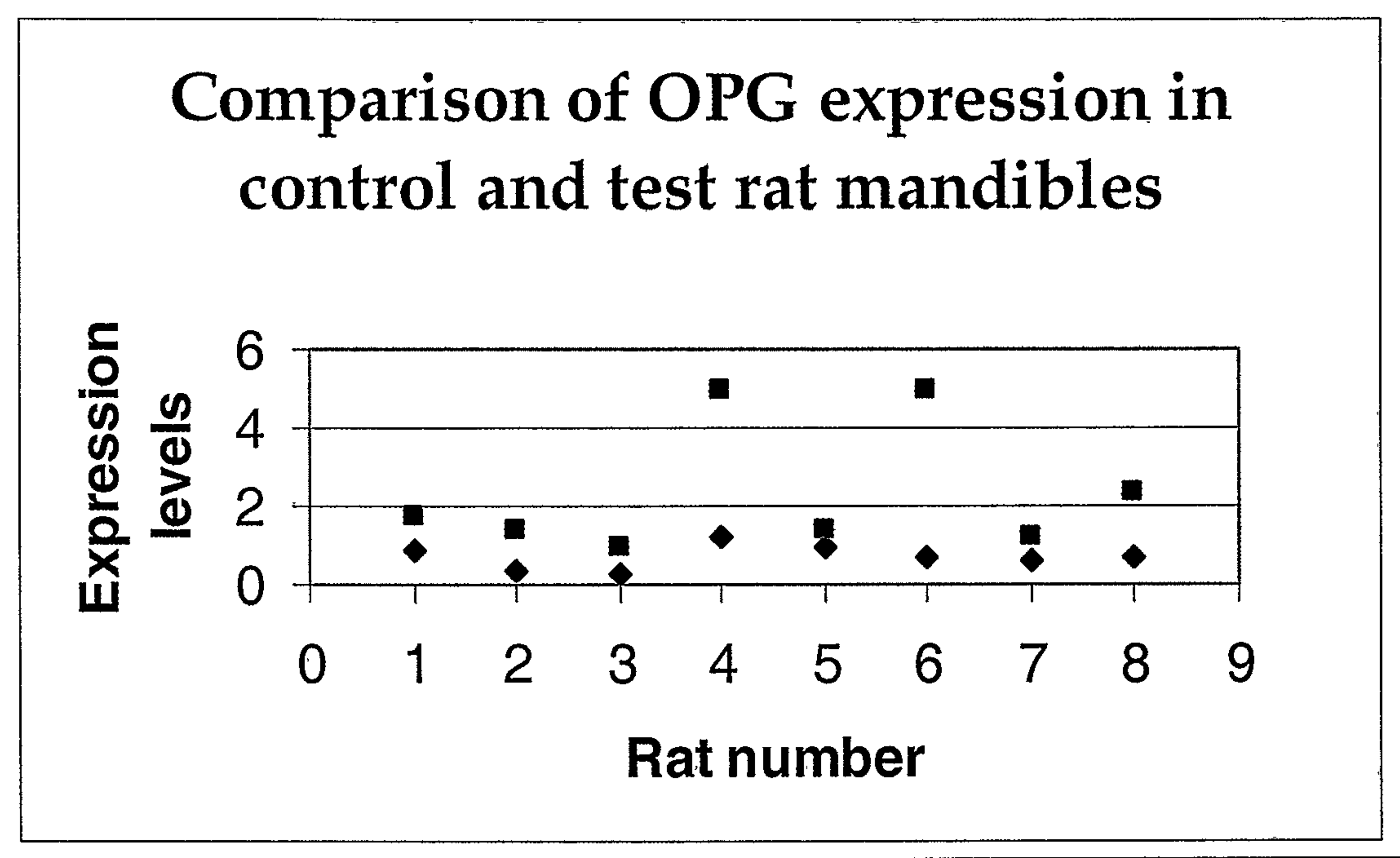
Legend:
Lane 1 molecular weight marker IV from Roche Diagnostics catalogue number 1449 460
Reference band used at 603 bp quantity assigned as 1

GelDoc analysis of OPG gene expression from control (right) side of rat mandibles

Description						
Track 1	Molecular weight marker					
Number		Height	Raw vol.	Quantity	Ratios of GAPDH/OPG	Ratios of OPG/ GAPDH
1		7057.42	1645997	1		
2	0					
Track 2	Positive control UMR106 cells					
Number		Height	Raw vol.	Quantity		
1	GAPDH	13881.69	3830071	2.3269		
2	OPG	21432.52	5034459	3.058607	0.75	1.3
Track 3						
Number		Height	Raw vol.	Quantity		
1	GAPDH	15014.09	4367557	2.653442		
2	OPG	15736.55	4151743	2.522327	1.04	0.9
Track 4						
Number		Height	Raw vol.	Quantity		
1	GAPDH	18426.23	5400531	3.281009		
2	OPG	2769.802	2021965	1.228413	2.6	0.38
Track 5						
Number		Height	Raw vol.	Quantity		
1	GAPDH	19290.63	5425142	3.295961		
2	OPG	2833.65	1426352	0.866558	4	0.25
Track 6						
Number		Height	Raw vol.	Quantity		
1	GAPDH	16128.36	4034447	2.451066		
2	OPG	19129.09	4802158	2.917477	0.8	1.25
Track 7						
Number		Height	Raw vol.	Quantity		
1	GAPDH	28776.3	6933659	2.212437		
2	OPG	13509.86	3867094	2.349393	1	1
Track 8						
Number		Height	Raw vol.	Quantity		
1	GAPDH	43227.07	10180983	6.185298		
2	OPG	29425.54	6779392	4.118715	1.4	0.7
Track 9						
Number		Height	Raw vol.	Quantity		
1	GAPDH	40310.55	9243723	5.615881		
2	OPG	20381.9	5065990	3.077764	1.5	0.6
Track 10	Negative control no expression seen					

Legend:
Lane 1 molecular weight marker IV from Roche Diagnostics catalogue number 1449 460
Reference band used at 603 bp quantity assigned as 1

Comparison of OPG expression levels between test and control rats



Legend:

- Test rat mandibles
- ◆ Control rat mandibles
- Lane 8 is average value for test and control

Table of results of OPG expression levels from Control and Test rat mandible tissue
As illustrated in Manuscript(see table 2 of Manuscript)

Rat Numbet	Control OPG	Test OPG
1	0.9	1.7
2	0.38	1.4
3	0.25	1
4	1.2	5
5	1	1.4
6	0.7	5
7	0.6	1.2
8	0.718571	2.385714

RANKL amplification form two Rat mandibles

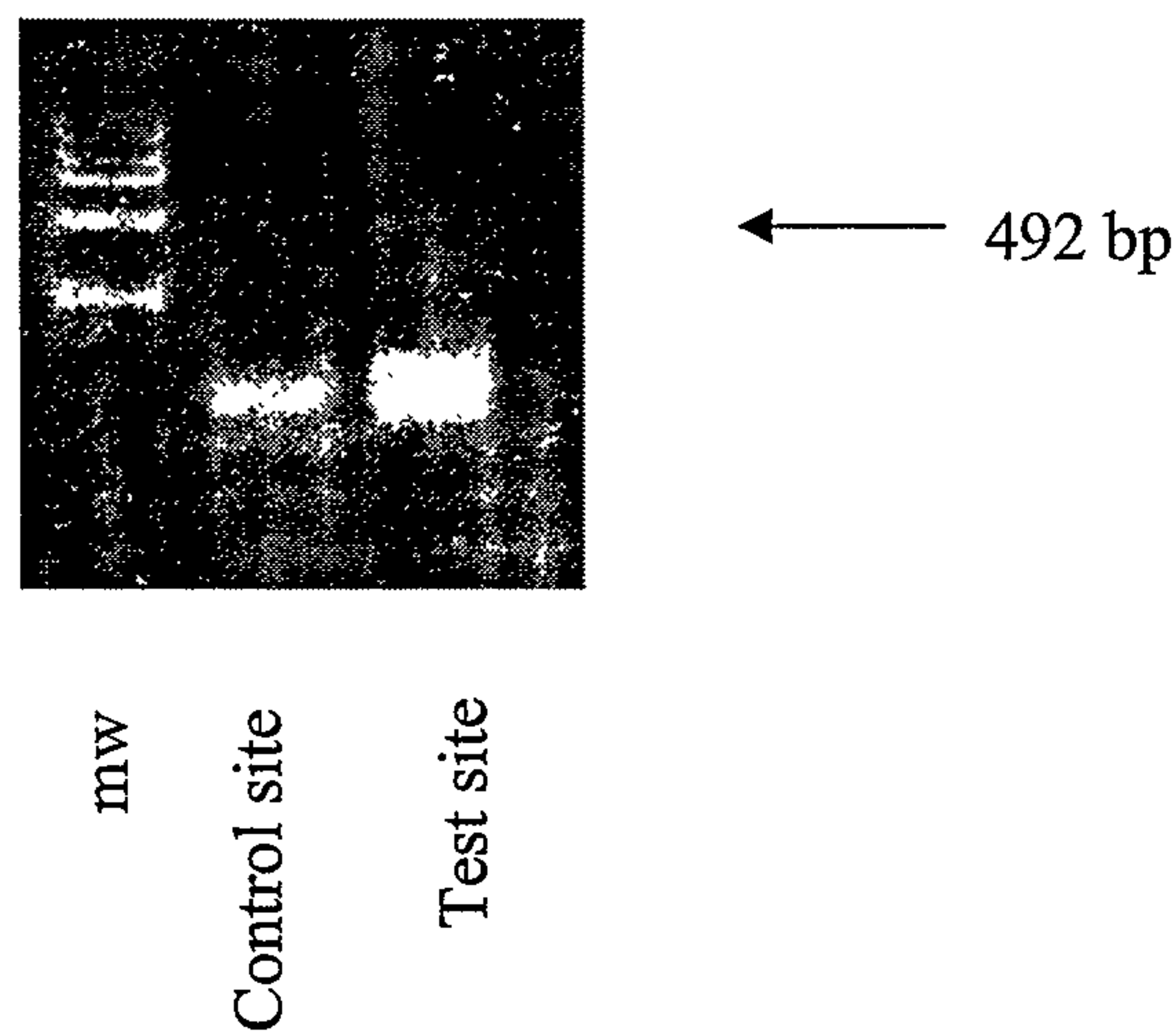


Figure shows agarose gel electrophoresis of RT-PCR of RANKL from test and control sites of RAT Number 2
Lane 1 is molecular weight marker IX
Lane 2 – amplification of mRNA from control site no amplification is seen
Lane 3 – amplification f mRNA from test site showing amplification product of 492 bp

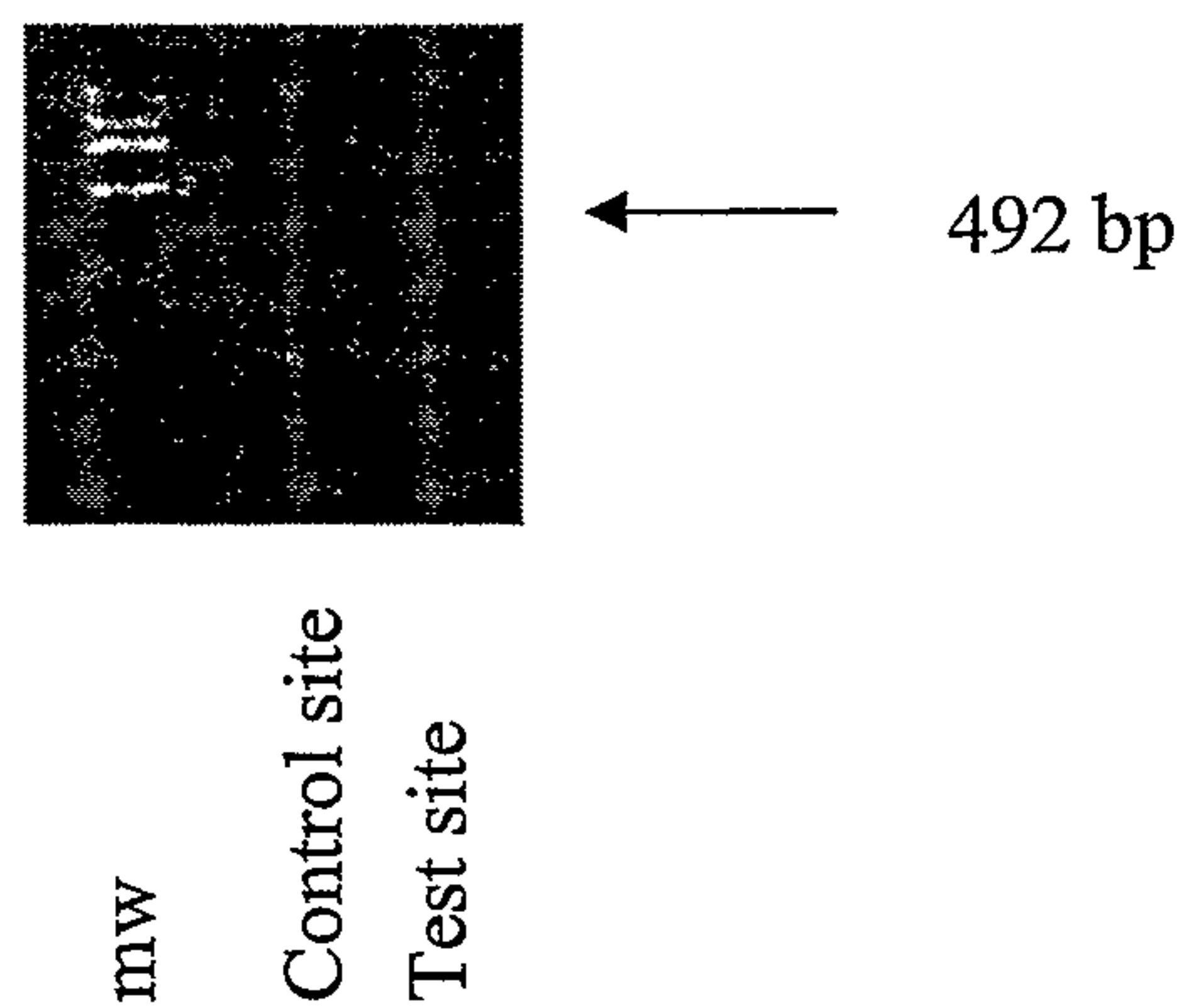


Figure shows agarose gel electrophoresis of RT-PCR of RANKL from test and control sites of RAT number 3

Lane 1 is molecular weight marker IX

Lane 2 – amplification of mRNA from control site no amplification is seen

Lane 3 – amplification of mRNA from test site showing amplification product of 492 bp

Automated sequencing of PCR product of OPG to confirm amplification of correct gene product



Model 3100
Version 3.7
Basecaller-3100POP6SF30_OPG
EC 1.5.0.0

1_30_OPG_F04_12.ab1
Eva Low/IDR
Basecaller-3100POP6SF30_OPG
Cap 12

Signal G:124 A:96 T:108 C:55
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WMM
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